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ORIGINAL ARTICLES

GRADED RESPONSE OF EXPERIMENTAL SKIN WHEAL, AND ITS SUPPRESSION BY CHLORPROMAZINE AND ANTIHISTAMINE

J. Collins, M.D., E. Dundas, B.Sc., M.D.,** H. Edgar, M.D.,****
*and J. H. Toogood, M.D., F.A.A.A., F.R.C.P.(C),**** London, Ontario*

INTRODUCTION

CLINICAL OBSERVATION, by the senior author, of conspicuous clinical improvement following the exhibition of chlorpromazine (Thorazine, Largactil)[1] in some patients with chronic urticaria prompted a study of the drug's anti-whealing properties in man. The results of some of the initial stages of this work are reported here.

Histamine was chosen as the whealing agent, in preference to a histamine-releasing drug or antigen, because we expected to encounter drug effects of a low order of activity, if any. It has been well established that the antagonistic effects of antihistamine drugs can be demonstrated much more easily with techniques utilizing "extrinsic" synthetic histamine than with techniques utilizing "intrinsic" histamine, that is to say, histamine released within or from the cell by some such stimulus as antigen-antibody union. The latter can be done, but often only by increasing the antihistaminic dose to much higher and less specifically active concentrations.[2]

Preliminary reports, presented to the American Academy of Allergy in 1957 and 1958, and the Royal College of Physicians and Surgeons of Canada in 1960, included portions of this work.

This work has been carried on by undergraduate medical students of the University of Western Ontario in the Allergy Laboratory of Victoria Hospital and has been made possible by the summer research fellowship plan of the Allergy Foundation of America. Additional grants, which are gratefully acknowledged, have been made by the Allergy Laboratory, Victoria Hospital, London (1957, 1958, 1959); Schering Corporation Limited, Montreal (1957); and Pfizer Canada Limited, Montreal (1959).

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**Allergy Foundation of America Fellow 1958.

***American Foundation of Allergic Diseases Fellow 1957.

****Instructor in Medicine, University of Western Ontario Medical School; Director, Allergy Laboratory, Victoria Hospital, London, Canada.

The histamine-induced experimental skin wheal in vivo was used as the basis for these studies, since it seems to be more closely comparable to the clinical entity in which we are interested, urticaria, than are some of the other phenomena resulting from the physiologic action of histamine. It is known that wide variation in the relative effects of different drugs can be demonstrated with respect to different components of the action of histamine in any one species: vascular versus dermal versus gastric acid release.[3]

Furthermore, human experimental subjects were included because of the wide qualitative and quantitative variations in the response to different drugs —including antihistaminic agents in particular—known to exist between species in various experimental in vitro and in vivo techniques employing a variety of lower animals.[3] In dealing with drugs in which the prime interest lies in their effects in man, a more meaningful expression of relative potency can be obtained if the experimental studies are performed on man; this applies nowhere more strongly than in the field of studies on histamine.

For these same reasons, we considered the earlier pharmacologic studies,[4, 5] which reported a negligible or extremely weak antihistaminic activity of chlorpromazine, to have limited value for our particular purposes since these reports were based on data derived from in vitro and in vivo lower animal techniques utilizing smooth-muscle-mediated phenomena, such as, aerosol-induced asthma and ileum contraction.

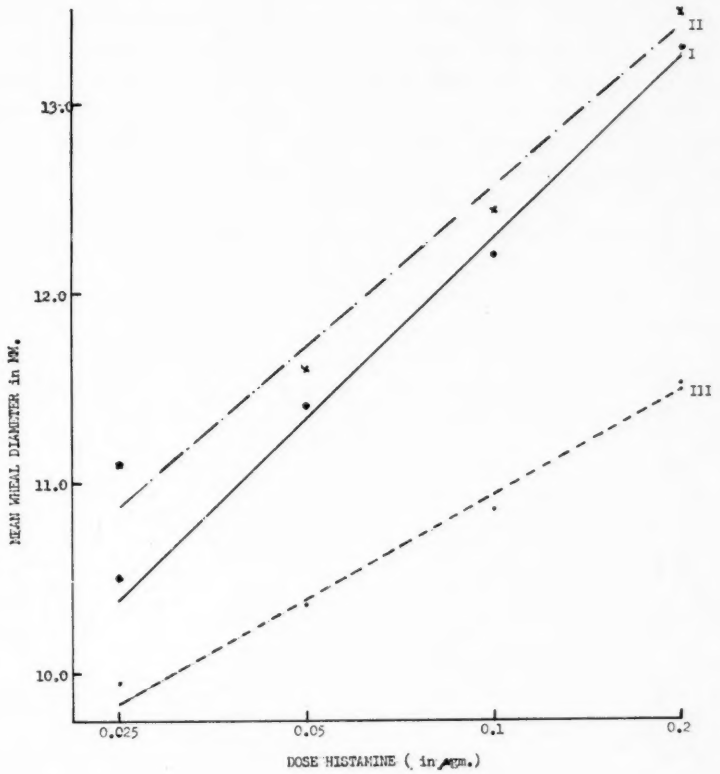
GENERAL METHOD

Histamine was used as the whealing agent. A series of dilutions in isotonic sodium chloride were prepared from ampules of histamine diphosphate (Abbott), containing 1 mg. per cubic centimeter of the histamine base. Selected Cooke-type syringes were used to inject 0.05 c.c. intradermally. The long and short diameters of the resulting wheals were measured with a vernier caliper to 0.01 mm., fifteen minutes after injection in the dog and ten minutes after injection in the human being. This mean wheal diameter provides a quantitative measure of response and thus permits application of statistical tests of the "mensuration" type. In general, these are preferable to the "enumeration" tests applicable to the analysis of data derived from end-point titration techniques, which provide an all-or-nothing, that is, a qualitative type of response. The variance due to experimental error was assumed to affect all parts of the experiment equally and was exactly accounted for by appropriate statistical analysis. One major source of variance in the size of the skin test response is the site into which the test injection is made.[6, 7] This body-site variation was eliminated in the design of the individual experiments which follow. Standard statistical techniques for tests of significance of results were employed.[8, 9]

Experiment 1.—Twenty-four skin-test sites were delineated on each side of the back of a dog. Graduated histamine test doses were given in a manner which permitted each test strength to be injected at each site the same number

of times. The results are shown in tabular form for one dog (Table I) and in graphic form for this and two additional dogs (Fig. 1).

Results: A linear log-dose response relationship is demonstrated. With this technique it is not difficult to obtain quite satisfactory correlation coefficients. The experimental results are consistently reproducible, since the same (qualitative) results have been obtained by three different experimenters using



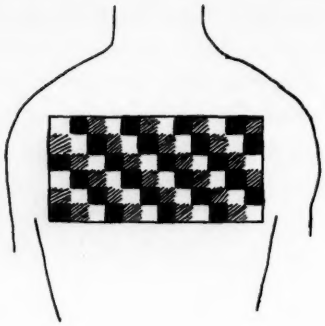
Dog	Experimenter	Regression Coefficient	Correlation Coefficient
I #1 '57	H.E.	3.056	0.998
II #1 '58	T.D.	2.658	0.986
III #1 '59	J.C.	1.734	0.988

Fig. 1.—Histamine wheal in dog. Log dose histamine versus mean wheal response. Doses used: 0.000025, 0.00005, 0.0001, and 0.0002 mg.

TABLE I. HISTAMINE WHEAL IN DOG*

HISTAMINE DOSE (GAMMA)	MEAN WHEAL DIAMETER (MILLIMETERS)
0.2	11.52
0.1	10.89
0.05	10.37
0.025	9.95

Error of estimate of regression line: ± 0.105.
*Dog 1, 1959; 48 tests per dose.



■ = 0.001 mg.

□ = 0.0005 mg.

▨ = 0.00025 mg.

Fig. 2.—Skin-test dose of histamine (milligram of base).

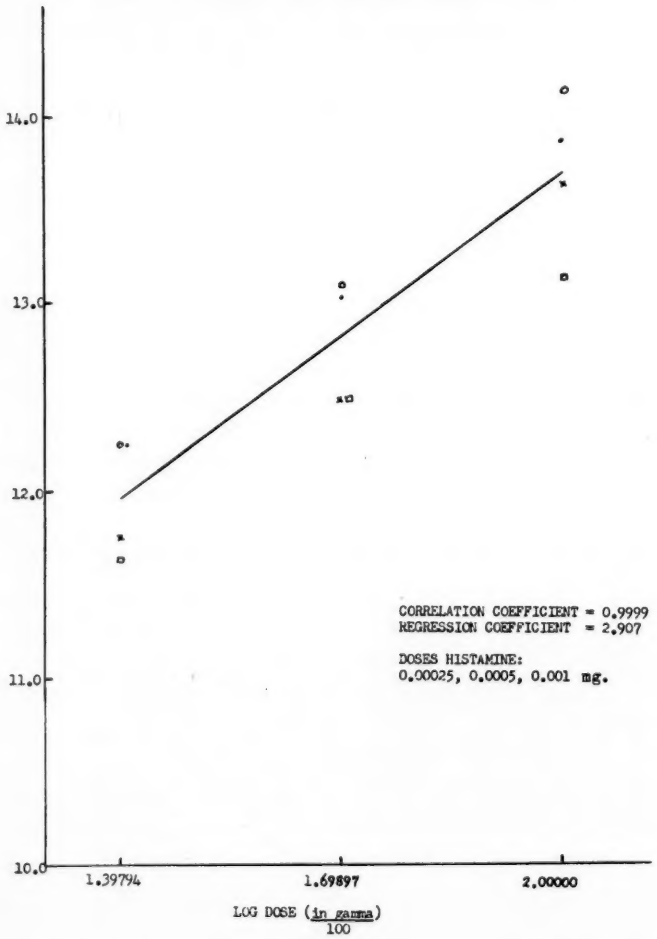


Fig. 3.—Histamine wheal in man. Log dose histamine versus mean wheal response in four human subjects

three different dogs. The difference between the regression coefficients describing the slopes of the three lines shown is to be expected, since the experimental conditions varied slightly from one animal to another.

Experiment 2.—Three graduated test doses of histamine were injected into four human beings according to the design shown in Fig. 2, which eliminates error due to body-site variation. Resulting data are plotted in Fig. 3.

Results: The same linear log-dose response relationship is demonstrated in human beings. Histamine shares this characteristic with a large number of pharmacologically active substances, that is, it follows the Weber-Fechner law. The influence of drugs or other factors on such phenomena may be manifested in an upward or downward shift of this line in response to enhancement or suppression, respectively, of the response under observation—in this case the skin wheal. A large number of statistical methods which will handle such data have been evolved.[8]

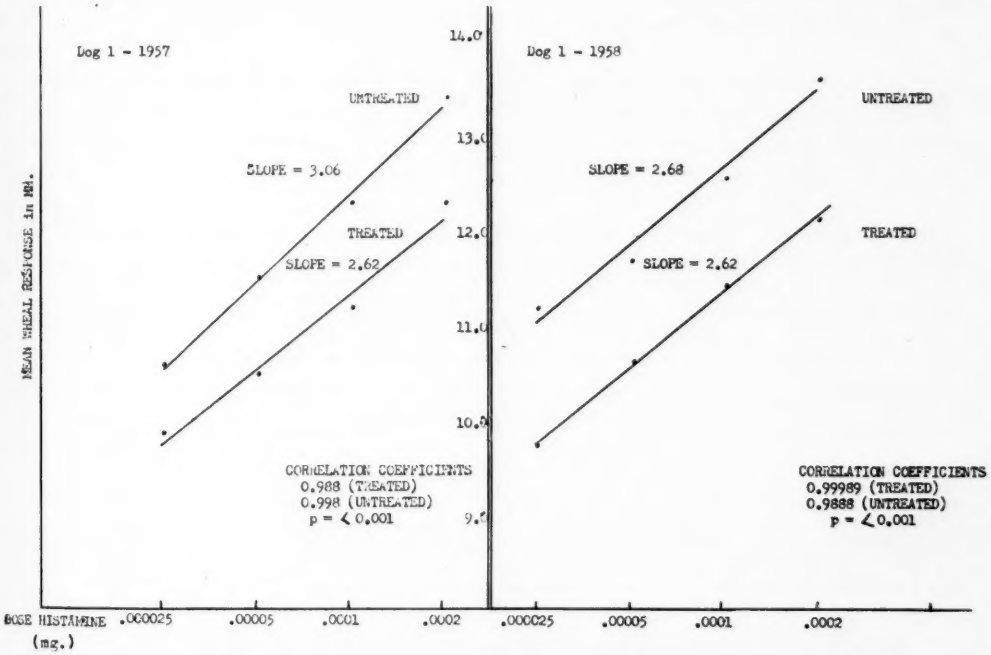


Fig. 4.—Log dose histamine versus mean wheal response in untreated dog and dog treated with chlorpromazine (150 mg.).

Experiment 3.—In order to determine whether chlorpromazine possessed any anti-whealing activity, dogs were tested in the manner just described before and after treatment with chlorpromazine.

Results: Results are outlined in Fig. 4 and demonstrate a significant decrease in wheal size following the chlorpromazine treatment.

Experiment 4.—To explore the relationship of drug dose to antiwhealing effect, four dogs were given four different doses of chlorpromazine. One testing

dose of histamine was used, 0.001 mg. of the base. Chlorpromazine doses were calculated on a milligram per kilogram of body weight basis and were injected intramuscularly two hours before the test. The actual doses that each dog received were staggered, according to the plan in Table II, so that no dog received an equivalent dose on any particular day and all received different dose sequences. In order to overcome the strong dose interaction effect that was evident by the time of the second testing, we lengthened the interval between tests from two to six days.

TABLE II. CHLORPROMAZINE DOSE ROTATION IN DOGS*

	DAY 1	DAY 3	DAY 23	DAY 29	DAY 35
Dog 1	0.75	1.50†	1.50	3.00	6.00
Dog 2	1.50	3.00†	3.00	6.00	0.75
Dog 3	3.00	6.00†	6.00	0.75	1.50
Dog 4	6.00	0.75†	0.75	1.50	3.00

*All doses in milligram per kilogram administered intramuscularly two hours prior to tests.

†Omitted from final calculations because of dose interaction.

As indicated in Table III, the third day's responses have been omitted from the calculations, since we believe that the above-mentioned carry-over effect plus a technical error invalidates a comparison of these data with the remainder of the experiment. Dog 2 had to be dropped from the experiment.

TABLE III. HISTAMINE WHEAL IN DOGS TREATED WITH CHLORPROMAZINE*

	0.75 MG./KG. (MM.)	1.5 MG./KG. (MM.)	3.0 MG./KG. (MM	6.0 MG./KG. (MM.)
Dog 1	11.1	12.08 10.28†	10.84	10.63
Dog 2		11.4	11.3†	
Dog 3	11.91	11.91	11.18	11.44 9.62†
Dog 4	12.19 8.63†	11.5	11.26	8.98
Average	11.7	11.7	11.1	10.4

*Test strength 0.0001 mg. histamine base.

†Omitted from final calculations because of dose interaction.

Results: Fig. 5 shows an increasing wheal-depressant effect in the dog as doses of chlorpromazine are increased above 1.5 mg. per kilogram. At and below this dose level, no drug effect is evident. A decrease of the wheal size by 11.11 per cent is produced by 6.0 mg. per kilogram of chlorpromazine administered intramuscularly.

Experiment 5.—Preliminary unpublished experiments by Dr. H. Edgar showed the same wheal depression in man following chlorpromazine treatment. Four human volunteers were therefore tested in the manner just described prior to and following administration of an antihistamine (chlorphenamine maleate*) and then of chlorpromazine. Resulting data are plotted in Fig. 6. It should be

*Chlor-Trimeton, Chlor-Tripolon.

noted that the antihistamine and the ataractic were given orally and in doses in the upper range of those generally recommended by the manufacturer for ordinary clinical usage. These drug doses were chosen to provide a basis for comparison of experimental results which would be more clinically meaningful than a strict milligram-for-milligram comparison.

Results: The experimental skin wheal is diminished in size following pretreatment with both chlorpromazine and chlorphenamine maleate. The difference between each of the treated states and the control is statistically significant

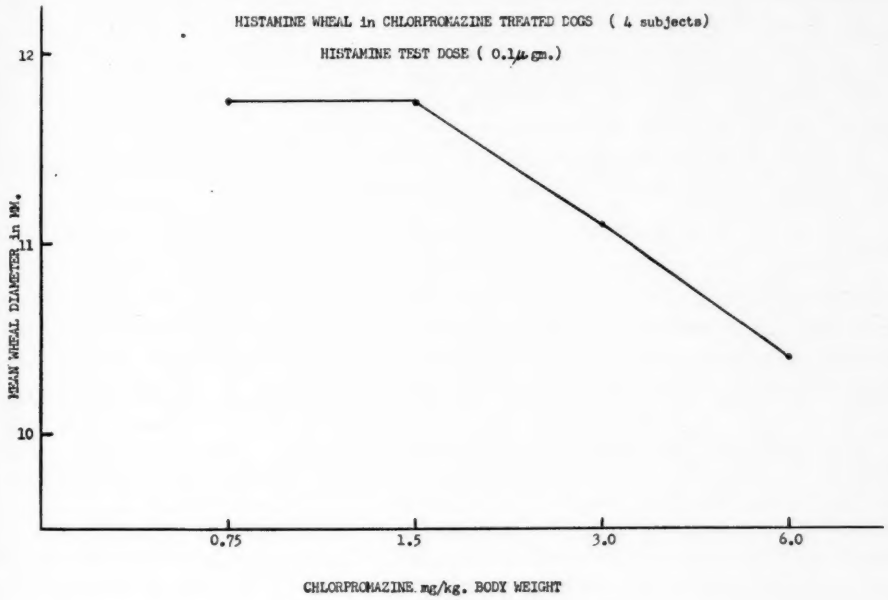


Fig. 5.—Histamine wheal in dog.

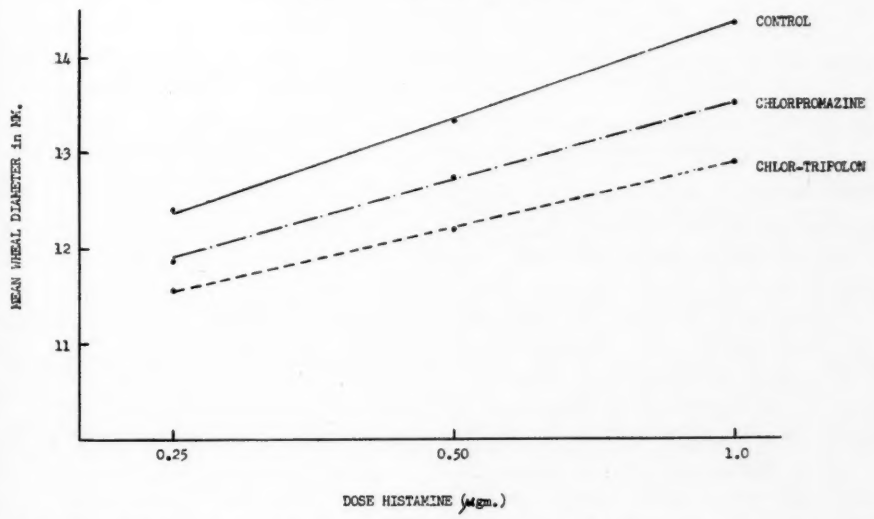


Fig. 6.—Histamine wheal in man, control versus treated (average of four subjects).

($p = <.001$). The design of this particular experiment does not permit precise potency ratios to be calculated. The data indicate, however, that subsequent work might reasonably be expected to demonstrate a wheal-depressing potency of not greater than 4 per cent for chlorpromazine, approximately one-half that of chlorphenamine maleate.

DISCUSSION

This antiwhealing effect of the so-called tranquilizer might account wholly or in part for the clinical improvement which occasionally follows its exhibition in cases of chronic urticaria. In our experience, however, this clinical improvement is highly variable with respect to the effective dose required. It seems unlikely that the order of "antihistaminic" activity demonstrated here could account for the striking symptomatic remission that we have observed in occasional cases in which chlorphenamine maleate (twice as strong an antiwhealing drug) and/or adrenocortical steroids had previously failed.

SUMMARY

Treatment with chlorpromazine can be shown with this graded-response type of assay to produce a definite inhibiting effect on the histamine-induced skin wheal in the dog and in the human being. In the doses used, chlorphenamine maleate has a more marked wheal-suppressing effect than chlorpromazine.

Many colleagues have helped us with their kind interest, constructive advice, and cooperation. Among these, we especially wish to mention Professor Carol Buck and Mr. James Wanklin of the Department of Psychiatry and Preventive Medicine, University of Western Ontario Medical School; Dr. R. Fisher of the Dominion Government Science Service; Professor J. A. F. Stevenson, Department of Physiology, University of Western Ontario Medical School; and Miss Muriel Dennison, Technician, Allergy Laboratory, Victoria Hospital.

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STUDIES ON THE RELATIONSHIP OF THE RELEASE OF SEROTONIN AND HISTAMINE, BY CHEMICAL MEANS, TO ANAPHYLAXIS IN THE RABBIT

T. Phillip Waalkes, Ph.D., M.D., and Harriette Coburn, A.B., Bethesda, Md.*

KATZ[1] first showed that histamine was released from the formed elements of rabbit blood by antigen-antibody reaction in vitro. Subsequently, numerous investigators[2-4] have verified this finding. The histamine in rabbit blood has been shown to be released also by a variety of other substances, including trypsin,[5] dextran,[6] peptone,[7] certain organic chemicals,[8, 9] and egg albumin.[4, 10] In 1953, Schachter[11] reported that histamine was liberated during anaphylaxis in the rabbit. Recently, serotonin (5-hydroxytryptamine) was found to be released both in vitro from rabbit platelets following antigen-antibody reaction and in vivo during anaphylaxis in this animal species.[10] In addition, it was shown that platelets and leukocytes disappear transiently from the circulation after the intravenous injection of antigen into a sensitized rabbit. During anaphylaxis in the rabbit, platelets and white blood cells form clumps which, in turn, are trapped in the pulmonary vessels. Consequently, the amount of serotonin and histamine in the lung is increased.[12] The peak for the rise in the lung concentration of these amines was found to occur four minutes after the intravenous injection of the antigen. The formation of the platelet-leukocyte thrombi and the release of serotonin and histamine appear to be due to the direct action of the antigen-antibody complex.[12]

Recent observations indicate that, like the antigen-antibody reaction, glycogen causes the release in vitro of serotonin and histamine from normal rabbit blood.[13] Humphrey and Jaques[14] found that serotonin and histamine are liberated from normal rabbit platelets when purified antigen and antibody are added to normal plasma in which these platelets are suspended. The removal of calcium ions from the plasma prior to the addition of the antigen and antibody prevented the liberation of these amines. If EDTA (ethylenediamine tetraacetate) is used as the anticoagulant, when whole blood from a sensitized rabbit is incubated with antigen, the release of serotonin and histamine is completely inhibited. The use of EDTA as an anticoagulant for normal blood also prevents the liberation of these amines by glycogen.[13] When injected intravenously into rabbits, glycogen produces a marked drop in the whole blood concentration of serotonin and histamine, the release of these amines into the

From the Section of General Medicine, National Heart Institute, National Institutes of Health, U. S. Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

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*Present address: Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

plasma, and an increase in the amount of both amines in the lung. The changes in serotonin and histamine produced both in vitro and in vivo by antigen-antibody reaction, by glycogen, and by antigen-antibody complex precipitate are all similar and suggest a common mode of action.

There are a variety of compounds or materials which release histamine in animals and produce physiologic changes sometimes seen during allergic reactions. In the rabbit, any substance which might exactly reproduce anaphylaxis should be able to cause specific changes both in vitro and in vivo. In vitro, the release of both serotonin and histamine from platelets would occur when the substance is incubated with normal whole blood, and the release would be inhibited in the presence of EDTA. In vivo, after intravenous injection, the transient release of serotonin and histamine into the plasma would be found associated with a rapid drop in the whole-blood concentration of these amines and the sudden increase in their amount in the lung. The many substances which liberate histamine from blood and tissues have been listed by Tabor.[15] Some of these have been reported to cause an anaphylactoid response in a variety of animal species. The present investigation was designed to determine whether specific substances, capable of causing either anaphylactoid reactions or the release of histamine in animals, could reproduce in rabbits all the changes just described which occur during anaphylaxis. It was hoped that, by this means, a clearer understanding of the changes observed and of the chemistry involved during anaphylactic reactions might be obtained.

MATERIALS AND METHODS

Male white rabbits (2.5 to 3.0 kilograms) were used in this study. Those rabbits that were used for antigen-antibody reaction studies were sensitized to horse serum as previously described.[10] Blood for the in vitro studies was obtained by cardiac puncture. Heparin or EDTA (10 per cent by volume of 1 per cent solution in isotonic saline) was used as the anticoagulant. All the glassware used for handling the blood was siliconized. The incubations with whole blood were carried out at 37° C. for thirty minutes with 50 per cent by volume of an isotonic saline solution of sodium chloride, containing each substance. For the in vivo studies, blood was obtained by inserting a plastic tube into the carotid artery. Heparin was the anticoagulant. The compounds or substances to be studied were dissolved in isotonic saline solution and injected intravenously. Samples of blood were obtained just before and one-half, one, two, three, and four minutes after the injection. The rabbits were killed immediately by a blow on the head. The lungs were excised, pressed between porous paper to remove excess blood, and homogenized in 0.1 N HCl. Serotonin and histamine analyses in blood and tissues were done by the method of Weissbach, Waalkes, and Udenfriend.[16]

All chemicals were reagent grade.* Only those substances which, in the concentrations used, caused release of serotonin and histamine in vitro were considered for the in vivo studies.

*Compound 48/80 was generously donated by Burroughs Wellcome and Co., Inc., Tuckahoe, New York.

RESULTS

Table I shows the amount of platelet-bound serotonin and histamine released when rabbit whole blood is incubated with a variety of materials. Glycogen, starch, antigen-antibody complex precipitate, trypsin, and egg albumin, in

TABLE I. RELEASE OF SEROTONIN AND HISTAMINE IN RABBIT BLOOD IN VITRO*

SUBSTANCE USED	FINAL CONCENTRATION (MG./ML.)	PLASMA SEROTONIN (γ/ML.)			PLASMA HISTAMINE (γ/ML.)		
		TOTAL	SUBSTANCE ALONE	ACTUAL AMOUNT RELEASED	TOTAL	SUBSTANCE ALONE	ACTUAL AMOUNT RELEASED
Glycogen	1.3	6.8	0	6.8	4.0	0	4.0
Starch	1.3	4.0	0	4.0	2.8	0	2.8
Peptone	4	0.3	0.3	0	0.5	0.3	0.2
	8	0.7	0.6	0.1	1.8	0.9	0.9
Proteose peptone	8	1.0	0.3	0.7	2.8	1.8	1.0
Tryptone	4	0.3	0.2	0.1	1.6	0.8	0.8
	8	0.6	0.6	0	3.0	2.0	1.0
Egg albumin	3.3	3.4	0	3.4	2.1	0.1	2.0
Trypsin	0.2	1.6	0	1.6	1.2	0.1	1.1
	0.5	4.8	0	4.8	2.5	0.2	2.3
Compound 48/80	0.2	0.2	0	0.2	0.2	0.2	0
PVP	1.3	0.2	0.1	0.1	0.3	0.2	0.1
Antigen-antibody reaction	(1% horse serum in isotonic saline)	3.7	0	3.7	2.6	0.1	2.5
Antigen-antibody complex†	--	7.0	0	7.0	3.8	0	3.8

*Whole blood was incubated at 37° C. for thirty minutes with 50 per cent by volume of isotonic saline solution of each substance to give the final concentration as indicated. To determine the amount of serotonin and histamine in the substance alone, isotonic saline was substituted for blood. After centrifugation, 2.5 ml. of diluted plasma was analyzed for the amines. Each determination is the average of four or more different experiments.

†The amount of antigen-antibody precipitate used was obtained from 1.5 ml. of serum from a rabbit sensitized to horse serum.

addition to antigen-antibody reaction, caused the release of serotonin and histamine from the platelets into the plasma. Proteose peptone, peptone, and tryptone contain histamine or a histamine-like compound. Fluorescent material was also found in these substances and a reading was obtained, but no distinct activation or fluorescence curve or peak characteristic for serotonin was found. These protein derivatives appeared to be capable of liberating a small amount of histamine during the incubation. The concentrations of peptone and proteose peptone were the same as those used by Ungar.[7] Under the experimental conditions employed, neither Compound 48/80 nor PVP (polyvinylpyrrolidone) released serotonin or histamine into the plasma.

Previous work[10] with egg albumin showed that it liberated both the serotonin and histamine in normal rabbit blood in vitro. When the egg albumin solution was diluted with isotonic saline solution to 0.001 per cent, no release occurred. However, egg albumin in the latter dilution liberated serotonin and histamine from the blood of rabbits sensitized to this protein. The intravenous injection of a saturated solution of egg albumin into normal rabbits released little, if any, serotonin and histamine into the plasma.

To obtain the results shown in Table II, blood was drawn from a rabbit sensitized to horse serum. The presence of EDTA as the anticoagulant completely inhibited the liberation of both serotonin and histamine by glycogen, starch, egg albumin, or horse serum. The amount of these amines released by trypsin was reduced, but not completely, when EDTA was used instead of heparin.

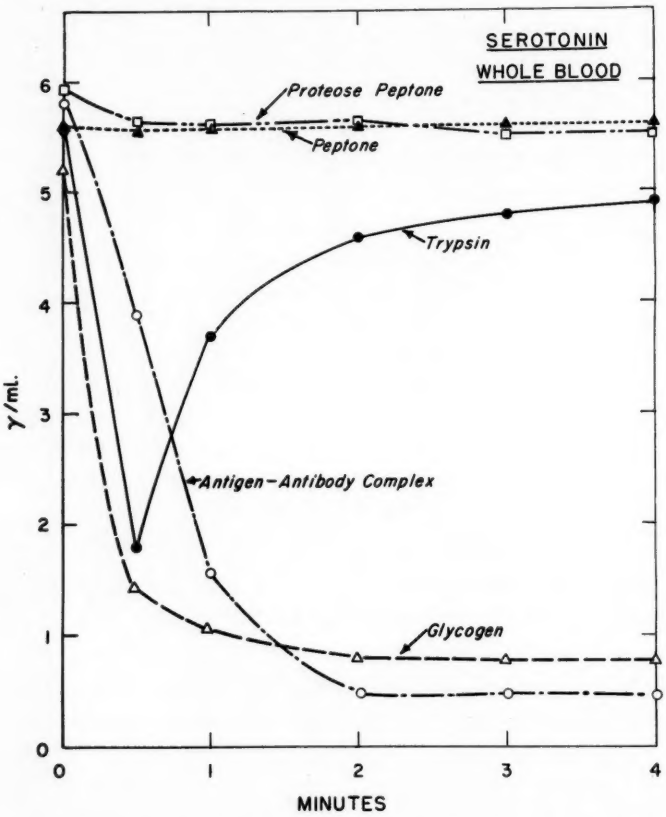

Fig. 1.—Serotonin in the blood of normal rabbits following the injection of either glycogen, crystalline trypsin, peptone, proteose peptone, or antigen-antibody complex precipitate. Whole-blood serotonin was determined before and after the intravenous injection into rabbits of the above substances in isotonic saline. The lung values for these same rabbits and the amounts of each substance used are given in Table III.

The whole-blood concentrations of serotonin and histamine before and after the intravenous injection of proteose peptone, peptone, trypsin, glycogen, and antigen-antibdy complex precipitate are shown in Figs. 1 and 2. Neither proteose peptone nor peptone caused any change in the content of these amines during the four-minute time interval. Trypsin lowered the whole-blood serotonin and histamine concentration, but a return to nearly normal levels occurred quickly. When greater amounts of trypsin were used (10 to 15 mg. per kilogram), marked intravascular clotting and death resulted within two to three minutes. Despite the use of heparin, clotted samples were always obtained.

Figs. 3 and 4 show that both serotonin and histamine are liberated in relatively large amounts by the antigen-antibody complex precipitate and also by glycogen. Trypsin caused the release of serotonin and histamine, whereas peptone or proteose peptone had little, if any, effect. A slight rise in the plasma histamine level following the intravenous injection of proteose peptone is presumed to be secondary to the histamine or histamine-like substance originally present in this material.

Table III gives the lung content of serotonin and histamine four minutes after the intravenous injection of the various materials studied. Neither proteose peptone nor peptone increased the amount of serotonin and histamine in

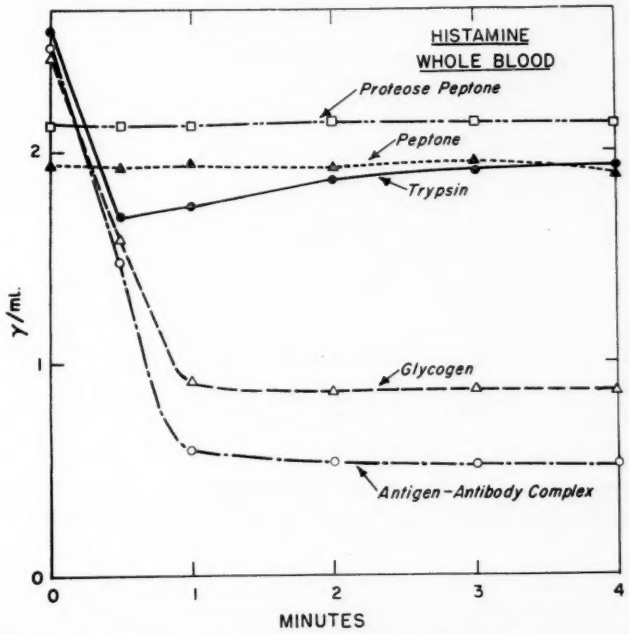

Fig. 2.—Histamine in the blood of normal rabbits following the injection of either glycogen, crystalline trypsin, peptone, proteose peptone, or antigen-antibody complex precipitate. Whole-blood histamine was determined before and after the intravenous injection into rabbits of the above substances in isotonic saline. The lung values for these same rabbits and the amounts of each substance used are given in Table III.

TABLE II. THE EFFECT OF HEPARIN OR EDTA ON THE IN VITRO RELEASE OF SEROTONIN AND HISTAMINE*

SUBSTANCE USED	FINAL CONCENTRATION (MG./ML.)	HEPARIN		EDTA	
		PLASMA SEROTONIN (γ/ML.)	PLASMA HISTAMINE (γ/ML.)	PLASMA SEROTONIN (γ/ML.)	PLASMA HISTAMINE (γ/ML.)
Glycogen	1.3	4.5	2.5	0.1	0.2
Starch	1.3	3.8	2.1	0.1	0.2
Trypsin	0.5	3.5	2.2	2.3	1.5
Egg albumin	3.3	3.1	2.1	0.1	0.2
Horse serum	(1%)	3.5	2.5	0.1	0.1
Isotonic saline	(control)	0.2	0.3	0.1	0.1

*Whole blood from a rabbit sensitized to horse serum was used and incubated for thirty minutes at 37° C. with 50 per cent by volume of an isotonic saline solution of the substance indicated. Either heparin or EDTA (10 per cent by volume of a 1 per cent solution) was used as the anticoagulant. After centrifugation, the diluted plasma was analyzed for serotonin and histamine. The results are the average from five or more experiments.

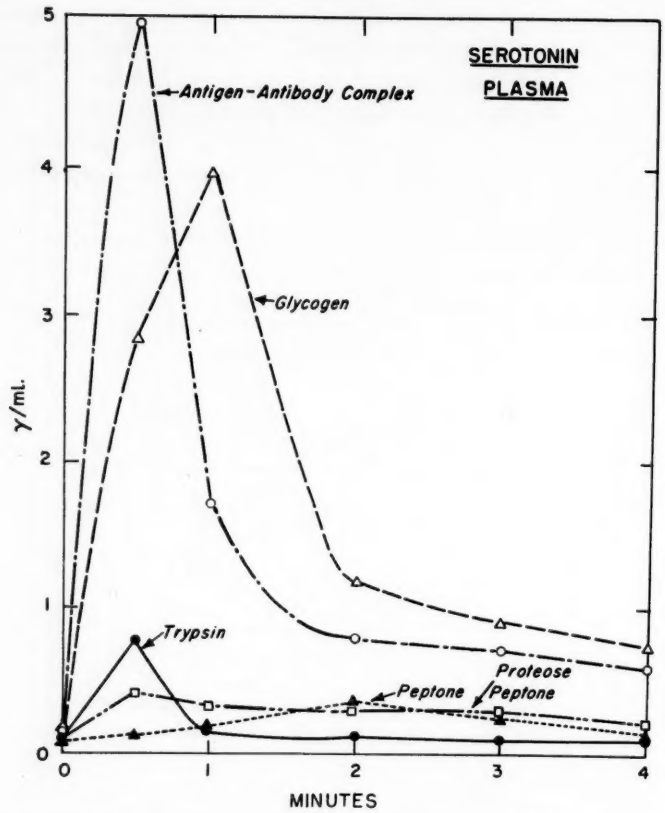

Fig. 3.—Serotonin in the plasma of normal rabbits after the injection of either glycogen, crystalline trypsin, peptone, proteose peptone, or antigen-antibody complex precipitate. Plasma serotonin was determined in the blood samples from which the data in Figs. 1 and 2 were obtained.

TABLE III. SEROTONIN AND HISTAMINE IN RABBIT LUNG*

SUBSTANCE INJECTED (MG. OR ML./KG.)	*Number animals used*	γ/GM.			
		SEROTONIN		HISTAMINE	
		AVERAGE	RANGE	AVERAGE	RANGE
Glycogen (100 mg.)	8	13.0	7.4-13.0	9.0	5.0-10.4
Trypsin (3-7 mg.)	5	15.8	5.0-20.0	9.1	6.3-12.0
Peptone (150 mg.)	5	2.3	1.5- 3.0	3.5	2.9- 5.0
Proteose peptone (150 mg.)	5	2.0	1.8- 2.8	5.2	3.8- 6.0
Antigen-antibody reaction (2 ml. horse serum)	6	21.5	17.0-25.3	14.3	6.6-22.0
Antigen-antibody complex†	3	20.8	18.0-23.2	13.9	11.5-16.2
Isotonic saline (2 ml.)	10	2.6	1.2- 4.6	4.5	3.0- 6.0

*Serotonin and histamine were determined in the lungs of rabbits that were killed by a blow on the head exactly four minutes after receiving intravenously an isotonic saline solution of the substance indicated. The lungs were immediately excised, pressed between porous paper to remove excess blood, and homogenized in 0.1 N Hcl.

†For each animal, the amount of precipitate injected was obtained from 12 to 15 ml. of serum from a rabbit sensitized to horse serum.

the lung. All the other substances injected, including glycogen, trypsin, and antigen-antibody complex precipitate, caused an elevation in the lung serotonin and histamine content similar to the rise found after the injection of antigen into a sensitized rabbit.

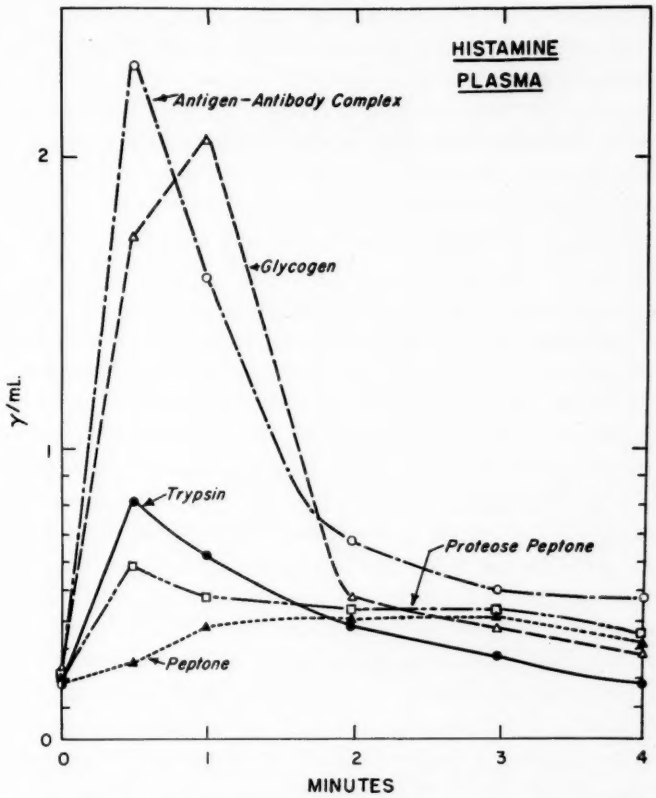

Fig. 4.—Histamine in the plasma of normal rabbits after the injection of either glycogen, crystalline trypsin, peptone, proteose peptone, or antigen-antibody complex precipitate. Plasma histamine was determined in the blood samples from which the data in Figs. 1 and 2 were obtained.

DISCUSSION

The changes in serotonin and histamine which occur following antigen-antibody reaction in the rabbit have been described.[10] All of these changes, including the release of serotonin and histamine into the plasma in vitro and in vivo and the decrease in whole-blood serotonin and histamine associated with a rise in the lung content of these amines in vivo, have been shown to be related to the specific action of the antigen-antibody complex.[12] Of all the substances employed in this study, only glycogen was found to reproduce most exactly the above changes as observed following antigen-antibody reaction or the use of the complex itself. Soluble starch in vitro caused the release of serotonin and histamine. Preliminary in vivo studies indicate that starch produces changes similar to those seen after the intravenous injection of glycogen. The

in vitro findings with these polysaccharides might be explained on the basis of the formation of an anaphylatoxin as described by Roche e Silva.[17] The immediate changes seen upon intravenous injection would be difficult to explain on this basis, however, and appear to be related to the direct action of glycogen or starch itself. It is of interest that dextran, after parenteral injection in the animals, causes reactions resembling allergic phenomenon.[18] In man, urticaria has been seen following the intravenous injection of dextran. The in vitro release of histamine in rabbit blood by dextran has been reported to be dependent on the molecular size as well as the concentration of this polysaccharide.[6] We have considered the possibility that structural changes in the glycogen molecule, produced during the isolation and purification procedures, may be a factor responsible for its ability to release these amines.[13]

The lack of effect of peptone or proteose peptone was surprising. In 1944, Ungar[7] reported that peptone in vitro caused the release of histamine in rabbit blood. The amounts, however, were small compared to those found in the present experiments with glycogen or antigen-antibody complex. The intravenous injection of peptone into animals has been shown to produce shock. In rabbits, intravenous injection of peptone has been followed by a drop in the whole-blood histamine which was secondary to a decrease in the number of platelets and leukocytes.[19] In dogs, the injection of 150 to 300 mg. of proteose peptone per kilogram has been reported to cause a similar drop in platelets and leukocytes.[20] Although several different samples of peptone and proteose peptone at 150 mg. per kilogram were used for intravenous injection in the present investigation, no changes resembling antigen-antibody reaction occurred. The whole-blood histamine and serotonin content remained the same during the period of time that blood samples were obtained. No increase in the amount of serotonin or histamine was found in the plasma, and the lung levels for these amines were normal. Perhaps impurities in the samples prepared and used several years ago, and not present in the current commercial material,* may have been responsible for the previous findings.

Compound 48/80 has been said to be effective as a histamine liberator in the cat, dog, rat, mouse, and man, but rather ineffective in the guinea pig.[21] Histamine is considered to be released due to the ability of Compound 48/80 to disrupt mast cells. In the present investigation, in vitro, at a concentration of 0.2 mg. per milliliter, no serotonin or histamine was released. In preliminary studies in which Compound 48/80 was given intravenously in amounts sufficient to cause death in the rabbit, no increase in plasma serotonin and histamine or decrease in the whole-blood content of these amines was found.

By in vitro studies, trypsin has been shown to cause the release of histamine in the blood of rabbits. When injected intravenously into rabbits in amounts of 3 to 7 mg. per kilogram, trypsin produced a fall in the whole-blood histamine concentration associated with a fall in the number of circulating white blood cells.[5] The present work with rabbits shows that trypsin releases both serotonin and histamine in vitro and in vivo. The use of EDTA as anticoagulant in vitro

*Peptone and proteose peptone obtained from Difco Laboratories, Inc., Detroit, Michigan.

completely inhibited the release of serotonin and histamine by antigen-antibody reaction, antigen-antibody complex precipitate, and glycogen, but the release of these amines by trypsin was only partially prevented. After the intravenous injection of trypsin, whole-blood serotonin and histamine values returned toward normal much more quickly than when antigen-antibody complex or glycogen is used or during antigen-antibody reaction. Similar to the findings during anaphylaxis, trypsin in vivo caused a release of serotonin and histamine and a rise in the lung content of these amines. Evidence has been presented previously to suggest that anaphylaxis depends upon the activation of a plasma protease.[19, 22] Other investigators have reported findings indicating that this is unlikely.[23, 24] From the results found here, trypsin appears to produce many of the changes seen during anaphylaxis in the rabbit, although not to the same extent as does glycogen. Trypsin also produces effects not seen during anaphylaxis.

Of interest is the ability of egg albumin, when incubated with normal rabbit whole blood, to release serotonin and histamine from the platelets. In addition, egg albumin causes anaphylactoid response in rats.[25] In view of the present findings with polysaccharides, the ability of egg albumin to release serotonin and histamine in vitro may be related to its glycoprotein structure.

The inhibition of release of serotonin and histamine in vitro when antigen is added to whole blood containing EDTA is probably secondary to the lack of ionized calcium.[14] Recently, Mongar and Schild[26] showed that the elimination of calcium ions inhibited histamine release from guinea pig lung during in vitro antigen-antibody reaction. Compound 48/80 and octylamine were active in liberating histamine from guinea pig lung despite removal of calcium ions by EDTA. Reserpine also releases serotonin and histamine in vitro from rabbit whole blood in the presence of EDTA as anticoagulant.[27] The findings suggest that the mechanism for release of these amines by the latter compounds is different from that for their release by antigen-antibody reactions or glycogen. The exact role of calcium in the release of serotonin and histamine from platelets has not been clarified.

The changes observed in platelets, serotonin, and histamine during anaphylaxis in a sensitized rabbit can be reproduced exactly by the intravenous injection of an antigen-antibody complex precipitate into a normal rabbit. These same changes can also be reproduced by the intravenous injection of specific polysaccharides into a normal rabbit. The nature of the relationship between the polysaccharide molecule and the antigen-antibody complex is not known and deserves further investigation.

SUMMARY

Anaphylaxis in the rabbit is accompanied by a drop in the whole-blood serotonin and histamine, a release of these amines into the plasma, and a rise in their concentration in the lung. When antigen is added to the blood from a sensitized rabbit, serotonin and histamine are released. If EDTA is used as

an anticoagulant, however, the release is prevented. In this study a variety of materials were tested, both in vitro with rabbit whole blood and in vivo by intravenous injection, to determine whether or not any of them could duplicate the findings which occur during anaphylaxis. Of all the substances employed, glycogen reproduced exactly the changes in platelets, serotonin, and histamine seen after the intravenous injection of antigen-antibody complex into normal rabbits or during anaphylactic reaction in this species.

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THE INFLUENCE OF HEPARIN ON THE BLOOD EOSINOPHIL

Solomon E. Barr, M.D., Halla Brown, M.D.,** and Robert F. Dyer, M.D.,****
Washington, D. C.

HEPARIN has many physiologic properties other than its well-recognized effect on blood coagulation. One of these properties is its ability to raise the blood eosinophil count.

Godlowski[1] first described this in 1951 when he demonstrated that dogs receiving huge intravenous doses of heparin exhibited an eosinophilia. He also showed that both human beings and dogs pretreated with heparin did not exhibit the usual eosinopenic response to ACTH, adrenal steroids, or insulin.[1] In 1956 and 1957, Hamilton and Lowenthal[2, 3] reported that the administration of large quantities of heparin could block the eosinopenia induced by steroids. In these experiments, huge doses of heparin (as much as 125,000 I.U. in a single injection) were given. However, Weissbecker and his coworkers[4, 5] have demonstrated that the administration of as little as 150 I.U. to a man can block the eosinopenic response to ACTH but not to cortisone or to epinephrine.

Recently we observed that some patients on maintenance therapeutic heparin showed an increase in eosinophil counts. We could find no report of the effect of long-term heparin therapy upon the blood eosinophil and therefore decided to investigate this phenomenon, using the guinea pig as a test subject.

Experiment 1 was a study of the effect of daily small doses of heparin upon the eosinophil count of the guinea pig over a ten-day period.

Eleven healthy female guinea pigs, each weighing from 410 to 728 grams, were selected. Six of these received daily intraperitoneal injections of heparin in the amount of 4 mg. (400 I. U.) per kilogram of body weight (0.4 c.c. of the 10 mg. per cubic centimeter of concentration per kilogram). Five control animals received daily intraperitoneal injections of 0.4 c.c. kilogram of heparin diluent (consisting mainly of isotonic saline). The two groups were treated in exactly the same manner except that one group received heparin and the other diluent.

Total eosinophil counts were obtained by nicking the ear veins with a hematology stylet, rapidly drawing the blood into two standard white cell pipettes, and diluting it with a solution containing propylene glycol, phloxine, and sodium carbonate. The pipettes were then shaken in an electric shaker, allowed to stand for two hours, and

From the Department of Medicine and The Allergy Clinic, the George Washington University Hospital, Washington 7, D. C.

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*Formerly, Resident in Medicine at the George Washington University Hospital; presently Resident in Allergy at the Roosevelt Hospital, New York, New York.

**Associate Clinical Professor of Medicine, the George Washington University School of Medicine; Chief, Allergy Clinic, the George Washington University Hospital.

***Formerly, Chief Medical Resident, the George Washington University Hospital.

reshaken. The cells were counted in four large counting chambers. Eosinophil counts were performed on each animal before the start of the experiment and were repeated on the fifth and the tenth days before the injection of heparin was given.

The animals that received heparin exhibited an average rise in eosinophil count during the first week of 69.5 cells per cubic millimeter, while those that received the saline diluent had an average fall of 71.8 cells per cubic millimeter, a difference of 141.3 cells per cubic millimeter (Table I). During the second week, the eosinophil counts were closer to their starting values. None of the results proved to be significant by analysis of variance.

TABLE I. EXPERIMENT 1—TEN DAYS OF TREATMENT WITH 4 MG. OF HEPARIN PER KILOGRAM

GUINEA PIG (ALL FEMALES)	EOSINOPHIL COUNTS		
	CONTROL	FIFTH DAY	TENTH DAY
Heparin-treated			
1	2.8	30.6	47.3
2	8.3	77.8	72.3
3	11.2	52.8	36.1
4	8.3	77.8	30.6
5	77.8	16.7	19.5
6	286.3	556.0	467.0
Averages	65.8	135.3	112.1
Diluent-treated			
1	27.8	8.3	61.2
2	19.5	5.6	8.3
3	300.2	19.5	119.5
4	141.8	50.0	36.1
5	11.2	58.3	22.2
Averages	100.1	28.3	49.5

Experiment 2 was identical to Experiment 1, except that the animals were treated with 10 mg. of heparin per kilogram of body weight instead of with 4 mg. per kilogram.

Twelve untreated healthy guinea pigs were selected. One half (four males and two females) received 10 mg. of heparin per kilogram of body weight (10 mg. per cubic centimeter) whereas the other six (four males and two females) received 1.0 c.c. per kilogram of saline diluent. The technique of injection was the same as in Experiment 1. Eosinophil counts were performed before the injections were begun, and on the fifth and eleventh days.

On the fifth day, the guinea pigs that received heparin exhibited an average rise of 114.0 eosinophils per cubic millimeter, while the control animals exhibited an average fall of 58.1—a total difference of 172.1 eosinophils per cubic millimeter (Table II). These results also did not prove significant by analysis of variance.

However, inspection of the results of these two experiments, considering only those animals with high initial counts (100 eosinophils per cubic millimeter or higher), revealed an interesting finding (Table III). All the animals receiving heparin showed a rise in eosinophil count, most marked on the fifth day, and all those receiving the saline control exhibited a fall in eosinophil count.

TABLE II. EXPERIMENT 2—ELEVEN DAYS OF TREATMENT WITH 10 MG. OF HEPARIN PER KILOGRAM

GUINEA PIG	SEX	EOSINOPHIL COUNTS		
		CONTROL	FIFTH DAY	ELEVENTH DAY
Heparin-treated				
1	M	33.4	30.6	38.9
2	M	411.4	519.8	525.4
3	M	16.7	89.0	13.9
4	M	19.5	8.3	43.4
5	F	97.3	27.8	58.4
6	F	308.6	895.2	403.1
	Averages	147.8	261.8	180.5
Saline-treated				
1	M	86.2	58.4	122.3
2	M	51.0	36.1	105.6
3	M	119.5	77.8	122.3
4	M	80.6	50.0	100.1
5	F	472.6	52.8	380.9
6	F	36.1	222.4	144.6
	Averages	141.0	82.9	162.6

TABLE III. ANIMALS IN EXPERIMENTS 1 AND 2 WITH INITIALLY HIGH COUNTS (100 EOSINOPHILS PER CUBIC MILLIMETER OR HIGHER)

TREATMENT	EXPERIMENT NUMBER	ANIMAL	SEX	EOSINOPHIL COUNTS		
				CONTROL	FIFTH DAY	CHANGE
Group A						
Heparin	1	6	F	286.3	556.0	+269.7
Heparin	2	2	F	411.4	519.8	+108.4
Heparin	2	6	M	308.6	895.2	+586.6
			Averages	335.5	657.0	+321.5
Group B						
Diluent	1	3	F	300.2	19.5	−280.7
Diluent	1	4	F	141.8	50.0	− 91.8
Saline	2	3	M	119.5	77.8	− 41.7
Saline	2	5	F	472.6	52.8	−419.8
			Averages	258.5	50.0	−238.5

TABLE IV. EXPERIMENT 3—THE EFFECT OF ONE INJECTION OF HEPARIN IN THREE HOURS UPON ANIMALS WITH INITIALLY LOW EOSINOPHIL COUNTS (LESS THAN 100 PER CUBIC MILLIMETER)

GUINEA PIG	SEX	EOSINOPHIL COUNTS	
		CONTROL	3 HR. POST-INJECTION
Heparin-treated			
1	F	16.7	19.5
2	F	30.6	25.0
3	F	38.9	8.3
4	F	66.7	158.5
5	M	38.9	36.1
	Averages	38.4	49.5
Saline-treated			
1	F	25.0	63.9
2	F	36.1	79.5
3	F	44.5	61.2
4	F	25.0	38.9
5	M	58.4	91.7
	Averages	37.8	67.2

Statistical evaluation by analysis of variance indicated that for these animals the difference between the effects of heparin and saline was significant (P less than 0.001).

Experiments 3 and 4 tested the effect of one injection of heparin upon the eosinophil count of guinea pigs three hours after administration. In Experiment 3 all the animals had initial eosinophil counts of less than 100 per cubic millimeter; in Experiment 4 all had counts above this figure. The injection technique and dosage were the same as in Experiment 2.

In Experiment 3 there was no difference between the eosinophil responses of the heparin-treated and the saline-treated groups (Table IV). However, in Experiment 4 (the group with initially high counts), there was a considerable difference (Table V). Those animals treated with heparin exhibited a rise of 108.8 cells per cubic millimeter, while those treated with saline revealed a fall of 191.4 cells per cubic millimeter. The results were not statistically significant.

TABLE V. EXPERIMENT 4—THE EFFECT OF ONE INJECTION OF HEPARIN IN THREE HOURS UPON ANIMALS WITH INITIALLY HIGH EOSINOPHIL COUNTS (100 PER CUBIC MILLIMETER OR HIGHER)

GUINEA PIG	SEX	EOSINOPHIL COUNTS	
		CONTROL	3 HR. POST-INJECTION
Heparin-treated			
1	F	864.6	556.0
2	F	1723.6	1921.0
3	M	573.2	1070.3
4	M	114.0	336.4
5	M	114.0	49.9
	Averages	677.9	786.7
Saline-treated			
1	F	264.1	253.0
2	F	433.7	514.3
3	M	1998.8	555.0
4	M	178.0	478.2
5	M	119.5	236.3
	Averages	598.8	407.4

DISCUSSION

The data indicated that heparin administered daily in small doses was able to produce an increase in the total eosinophil count in those guinea pigs that had some degree of eosinophilia at the beginning of the experiment. This effect was more evident on the fifth day than on the tenth or the eleventh day after the initiation of heparin injections. No effect was apparent during the experiment in which the eosinophil count was performed three hours after an injection. Control animals, treated in exactly the same way except for the substitution of saline for heparin, revealed a fall in total eosinophil count.

The reason for the development of spontaneous eosinophilia in guinea pigs is not known. Guinea pigs tend to develop an eosinophilia as they grow larger, perhaps as the result of infestation by such parasites as *Megastoma entericum* in the small intestine and Infusoria in the cecum.[6] The fall in

eosinophil count in the control animals may result from the trauma and excitement of daily handling causing increased secretion of epinephrine and steroids.

Our results differ from those of Samter,[7] who has reported that heparin administration does not produce a significant eosinophil rise in the guinea pig. However, his technique differed from ours. (1) He used small guinea pigs of 400 to 450 grams, whereas we used larger animals and noted the eosinophil response to be significant only in those animals whose counts were initially high. (2) He utilized only the differential smear, but we used the more accurate total eosinophil count. (3) He waited seventy-two hours after a single heparin injection before obtaining smears, whereas in Experiments 1 and 2 we gave daily injections for five days before obtaining a count. (4) He used a dose of 1 mg. per guinea pig, whereas in our latter three experiments we gave an average of 5 mg. heparin to each guinea pig.[7]

The reason for this eosinophil response to heparin is not known. It is possible that the animals became hypersensitive to the heparin. Those guinea pigs with initially low eosinophil counts showed no rise in their counts, however, while those animals with initially high counts showed a significant rise in their counts. In allergy to heparin one would expect a similar rise in eosinophils in both groups. Cortez, Grolnick, and Loewe,[8] using the same brand of heparin as we used, were unable to sensitize guinea pigs.

Another possibility is that heparin mobilized eosinophils from the intestinal wall. This might explain the marked effect upon those animals with initially elevated eosinophil counts and presumed intestinal parasites. Godlowski[9] has reported that when heparinized blood was perfused through isolated dog intestine the number of eosinophils decreased in the intestinal wall and increased in the perfusing blood.

It is likely that heparin interferes in some way with the effect of hormones upon the eosinophil. Normally, ACTH, cortisone, and epinephrine produce a fall in the blood eosinophil count. However, Weissbecker and Schröter[5] reported that the prior intravenous administration of 400 I.U. of heparin will prevent the eosinopenic response to ACTH but not to cortisone or epinephrine. He postulated a direct ACTH-heparin antagonism. Supportive evidence for this is the report of Köhler, Loll, and Schröer[10] that ACTH can inhibit the anticoagulant action of heparin in vitro.

Other experimental results, however, conflict with the concept of a direct ACTH-heparin block. Normally, ACTH injection produces adrenal ascorbic acid depletion. Fekete[11] has reported that male white rats exhibited the same degree of ACTH-induced adrenal ascorbic acid depletion, whether they received heparin or not. Morris, Lowenthal, and Hamilton[12] have observed that in white rats heparin pretreatment did not affect the adrenal ascorbic acid depletion which results from injection of the stress-inducing agents, sodium salicylate, epinephrine, and histamine.

Heparin may directly interfere with the action of the adrenal steroids. Muehrcke, Lewis, and Kark[13] reported that incubation of human blood with cortisone could significantly reduce the eosinophil count but that this action

could be blocked by the addition of heparin to the mixture. However, neither Essellier and his colleagues[14] nor Godlowski[9] could confirm these results in vitro. That heparin pretreatment may block in vivo the action of adrenal steroids has been demonstrated by Godlowski in human beings and dogs,[1, 9] and by Hamilton and Lowenthal[2] in white rats. In 1957, Hamilton[3] reported that he could overcome the heparin block of the action of cortisone upon the white rat leukocyte by increasing the dose of cortisone. He could then overcome the action of this larger dose of cortisone by increasing the amount of heparin administered. By giving the heparin after the adrenal steroids, he could still obtain the block, but only if larger doses of heparin were employed.

The difficulty with accepting cortisone-heparin antagonism as a naturally occurring physiologic reaction lies in the huge doses of heparin necessary in these experiments. Godlowski[1, 9] noted a block only if he used 1,950 I.U. per kilogram intravenously in human beings and dogs. In Hamilton's[3] experiments, the smallest amount of heparin found effective was 10 mg. (1,000 units) per 100 grams of body weight of the rat. In his experiments, 3 mg. (300 units) of heparin per 100 grams of body weight would block the rat's leukocyte response to epinephrine, salicylates, and ACTH but not to cortisone.[2]

Another possible explanation is that, rather than inhibit the ACTH and steroids per se, the heparin may block those enzymes producing eosinolysis in response to the steroids:

$$\text{ACTH} \rightarrow \text{adrenal steroids} \rightarrow \text{enzymes} \rightarrow \text{eosinophil.}$$

If this theory were correct, one would expect that the eosinopenic actions of ACTH and of the adrenal steroids would be blocked by equivalent doses of heparin. Instead, experiments have rather consistently shown that the dose of heparin necessary to inhibit cortisone is much larger than that necessary to inhibit ACTH.

SUMMARY AND CONCLUSIONS

The fact that heparin may produce an effect on the blood eosinophil has been known since 1951. Prompted by the observation that certain patients on maintenance heparin therapy appeared to exhibit an eosinophilia, we investigated the action of daily heparin injections on the eosinophil count in guinea pigs. We found that the direct eosinophil count is an accurate and practical, although somewhat difficult, procedure to perform in these animals. Animals injected daily with heparin showed a rise in count. The effect was most consistent in those animals possessing a slightly elevated eosinophil count before injections were started, and it was more apparent on the fifth than on the tenth day of the experiment. The possible reasons for this reaction are discussed. Most of the reported experiments indicate that heparin blocks the action of some eosinolytic substance. The exact mechanism is unknown, although there is evidence to suggest that there is interference with the utilization of ACTH, adrenal cortical steroids, or both.

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THE SEPARATION OF SUBSTANCES IN TIMOTHY POLLEN EXTRACT PRODUCING ALLERGIC SKIN REACTIONS FROM THOSE PRODUCING HEMAGGLUTINATION REACTIONS

Arthur Malley, Arthur Lietze, Ph.D., and Charles E. Reed, M.D., Corvallis and Portland, Ore.*

DURING the past two years a major goal of our laboratory has been to isolate and characterize chemically one or more allergens from timothy pollen. In an attempt to find an in vitro assay for allergens we investigated the technique of Gordon, Rose, and Sehon[1, 2] of hemagglutination of rabbit red blood cells coupled to pollen extracts by a diazo bond. The present communication reports that hemagglutination may not be a suitable method for determination of allergic activity of various fractions of crude allergens.

MATERIALS AND METHODS

The hemagglutination technique of Gordon, Rose, and Sehon[2] was followed with these modifications. Whole timothy pollen and its fractions were diazotized to the rabbit red blood cells in a concentration of 60 mg. of organic solids per 100 ml. of solution. Serum was obtained from patients known to be sensitive to timothy pollen. Control serum was obtained from normal persons. We found that fresh human serum produces nonspecific hemolysis of diazotized rabbit red blood cells. Gordon, Rose, and Sehon prevented this hemolysis by heating the serum at 56° C. for thirty minutes. We were unwilling to heat the serum for fear of altering its skin-sensitizing activity, but we found that the hemolysis can also be prevented by allowing the serum to stand at room temperature for seventy-two hours, a procedure which did not destroy the skin-sensitizing capacity. Therefore, all hemagglutination titers were performed with serum which had been kept at room temperature for seventy-two hours and then frozen for storage. In the interpretation of the hemagglutination test, no tube was considered to show positive agglutination unless the cells were evenly dispersed over the entire surface of the bottom of the tube after the tubes had been sitting at room temperature for twenty-four hours. Hemagglutination titers are reported as the reciprocal of the maximum dilution yielding positive reactions. Unfortunately, not all determinations were carried out to negative reactions.

From the Department of Chemistry and the Science Research Institute, Oregon State College, Corvallis, Ore., and The Division of Allergy, University of Oregon Medical School, Portland, Ore.

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The antigen used was timothy pollen which was fractionated by ammonium sulfate at 4° C. Our fractionation procedure is diagrammed in Fig. 1. Each fraction was dissolved in 0.15 M phosphate buffer, pH 10.4, containing 10 per cent glucose and reprecipitated twice. The ammonium sulfate was later removed by dialysis for twenty-four to forty-eight hours against running tap water in Visking No. 24 cellophane tubing.

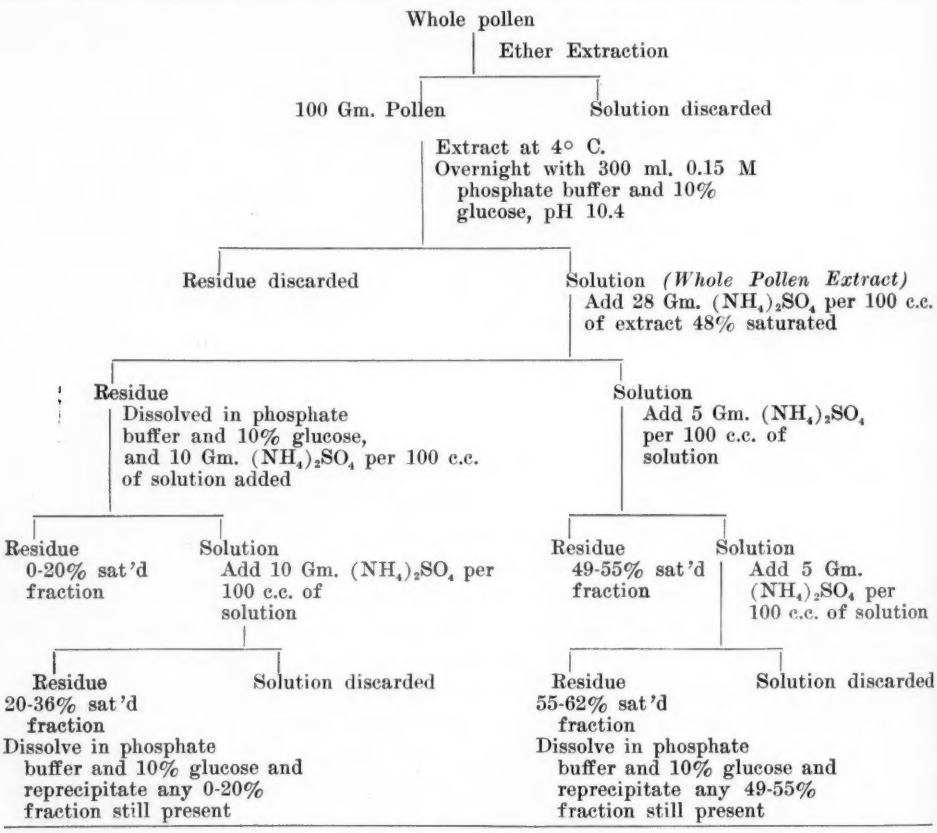
Whole pollen
Ether Extraction
100 Gm. Pollen
Solution discarded
Extract at 4° C.
Overnight with 300 ml. 0.15 M phosphate buffer and 10% glucose, pH 10.4
Residue discarded
Solution (Whole Pollen Extract)
Add 28 Gm. (NH4)2SO4 per 100 c.c. of extract 48% saturated
Residue
Dissolved in phosphate buffer and 10% glucose, and 10 Gm. (NH4)2SO4 per 100 c.c. of solution added
Solution
Add 5 Gm. (NH4)2SO4 per 100 c.c. of solution
Residue 0-20% sat'd fraction
Solution
Add 10 Gm. (NH4)2SO4 per 100 c.c. of solution
Residue 49-55% sat'd fraction
Solution
Add 5 Gm. (NH4)2SO4 per 100 c.c. of solution
Residue 20-36% sat'd fraction
Dissolve in phosphate buffer and 10% glucose and reprecipitate any 0-20% fraction still present
Solution discarded
Residue 55-62% sat'd fraction
Dissolve in phosphate buffer and 10% glucose and reprecipitate any 49-55% fraction still present
Solution discarded

Fig. 1.—Fractionation of timothy pollen. (The precipitation of pollen with ammonium sulfate was carried out at 4°C.)

Whole pollen (WP) and its fractions were assayed for total organic solid content by the dichromate reduction method of Johnson,[3] and then assayed for skin reactivity by intradermal injection of 0.02 ml. of decimal dilutions containing 10^{-4} to 10^{-8} mg. of total organic solids per milliliter of Coca's solution. Each fraction was assayed intradermally in ten patients known to be sensitive to timothy pollen. The skin test titer of each fraction was expressed as the reciprocal of the mean end point dilution in milligrams of total organic solids per milliliter. The end point was taken to be a wheal and/or erythema larger than the control test of Coca's solution.

RESULTS

The hemagglutination reaction was first carried out using whole water-soluble timothy pollen extract and serum of untreated timothy-sensitive patients. Normal sera gave negative controls, and the sera of timothy-sensitive patients gave weakly positive reactions (Table I).

TABLE I. HEMAGGLUTINATION TITERS WITH WHOLE POLLEN EXTRACT AND SERA FROM UNTREATED TIMOTHY-SENSITIVE PATIENTS

	HEMAGGLUTINATION TITERS
Allergic Sera	
R. D.	16
P. H.	4
K. S.	16
L. D.	4
R. T.	16
R. C.	16
G. B. W.	2
C. S.	16
B. B.	8
A. B.	8
L. H.	16
Normal Sera	
A. M.	0
A. L.	0
E. H.	0

We next carried out the hemagglutination reaction using ammonium sulfate precipitated fractions of timothy pollen and a serum from an untreated patient. This serum gave positive passive transfer reactions when diluted 1:10,000 and tested forty-eight hours later with 0.1 mg. of total organic solids per milliliter of whole pollen extract. We compared each fraction of ammonium sulfate precipitated pollen for its hemagglutination and skin reaction titer (Table II). The whole pollen skin test titer was 10^5. The fraction of whole pollen extract precipitated by 20 per cent saturated ammonium sulfate gave skin test titers of less than 10^4, but fairly strong hemagglutination titers. The fraction soluble

TABLE II. CORRELATION OF SKIN AND HEMAGGLUTINATION TITERS OF FRACTIONS OF TIMOTHY POLLEN

FRACTION	HEMAGGLUTINATION TITER	SKIN TITER
Whole pollen	32	10^5
0-20% saturated	64*	$< 10^4$
20-46% saturated	16	Negative
46-52% saturated	4	10^6
52-57% saturated	4	10^6
$>$ 57% saturated	4	$< 10^4$

*Maximum dilution carried out.

in 20 per cent but insoluble in 46 per cent saturated ammonium sulfate gave lower hemagglutination titers and no skin reaction at 10^4. Whereas the fraction soluble in 46 per cent but precipitated by 52 per cent saturated ammonium sulfate, and the fraction soluble in 52 per cent but precipitated in 57 per cent saturated ammonium sulfate gave skin test titers of 10^6, but low hemagglutination titers. The fraction soluble in 57 per cent saturated ammonium sulfate gave both low skin test titers and hemagglutination titers. From this experi-

ment it appeared that the antigen of the hemagglutination and the antigen of the skin reaction were not the same substance.

The results of further studies to clarify this difference are presented in Table III. The hemagglutination test was performed with whole pollen (WP), the 0-20 per cent fraction, and the 49-55 per cent fraction (the fraction shown to possess maximum skin test activity) using serum from untreated timothy-sensitive patients, from treated patients whose last timothy injection had been more than eighteen months earlier, and from patients who were currently receiving treatment with timothy pollen in a total dose range of 5,000 to 25,000

TABLE III. HEMAGGLUTINATION TITERS WITH FRACTIONS OF TIMOTHY POLLEN AND SERUM OF TIMOTHY-SENSITIVE AND NORMAL SUBJECTS

DONOR	TREATMENT STATUS	HEMAGGLUTINATION TITERS		
		WHOLE POLLEN	0-20% SATURATED	49-55% SATURATED
K. S.	Nontreated	4	4,096*	0
A. E.	Nontreated	32	3,072	32
J. R.	Nontreated	8	512*	8
D. R.	Nontreated	16	384	8
L. H.	Nontreated	8	64	4
C. S.	Nontreated	4	64*	0
R. D.	Nontreated	16	64	8
N. McC.	Treated 18 mo. previously	32	512*	64
R. T.	Treated 18 mo. previously	4	64*	0
R. C.	Treated 18 mo. previously	8	64*	4
L. D.	Treated 18 mo. previously	4	64*	0
T. S.	Treated 18 mo. previously	16	64*	32
N. C.	Treated currently	4	64	4
J. S.	Treated currently	16	64†	8
M. H.	Treated currently	32	4,096*	16
E. F.	Treated currently	8	128	8
C. F.	Treated currently	4	128	16
A. R. P.	Treated currently	4	3,072	0
R. D.	Treated currently	8	512*	32
A. K.	Treated currently	16	64	8
G. C. M.	Treated currently	4	2,048	0
A. C. H.	Treated currently	16	512	8
N. C.	Treated currently, serum heated	8	256*	8
A. K.	Treated currently, serum heated	16	256*	4
M. H.	Treated currently, serum heated	32	4,096*	16
A. R. P.	Treated currently, serum heated	4	4,096*	8
E. F.	Treated currently, serum heated	4	256*	4
J. S.	Treated currently, serum heated	4	512	4
A. S.	Normal subject	64	128	8
A. M.	Normal subject	0	16	0
B. H.	Normal subject	0	16	0
M. C.	Normal subject	0	0	0
K. R.	Normal subject	0	8	0
J. M.	Normal subject	0	4†	0
A. D.	Normal subject	0	0	0
M. N.	Normal subject	0	64†	0

*Maximum dilution carried out.
†Confusing reactions described in text.

protein nitrogen units (as well as an equal amount of other grass pollens). Several of the sera were tested after being heated at 56° C. for six hours to destroy the skin-sensitizing activity. The 0-20 per cent fraction gave strongly positive hemagglutination, whereas the 49-55 per cent fraction gave weakly posi-

tive reactions. A number of sera from individuals with no symptoms of hay fever or asthma during the grass season were also tested as normal controls. A few gave positive hemagglutination reactions, but most were negative.

The sera of a dust-sensitive and egg-sensitive patient were also tested and agglutination was not observed with any of the fractions tested. We concluded from these results that the hemagglutination observed with the 0-20 per cent ammonium sulfate fraction of timothy grass pollen is a specific phenomenon.

Several of the hemagglutination reactions giving alternating positive and negative tubes were confusing. For example, J. M. was positive at 4, negative to 64, positive to 256, negative again to 512, and positive at 2,048. One serum of this type was tested at a different time and gave essentially the same alternating positive and negative hemagglutination pattern. With reactions of this sort we have recorded the serum titer as being 4. Two strongly positive sera from treated patients gave prozone reactions.

The data showed clearly that the fraction possessing high hemagglutinating activity was distinct from the fraction possessing high skin test activity, but that the hemagglutination test both to the 0-20 per cent fraction and to the 49-55 per cent fraction distinguished the allergic from the normal sera with a fair degree of accuracy. The 0-20 per cent fraction makes up about 0.5 per cent of the total organic solid content of the whole pollen extract. The 49-55 per cent fraction makes up about 9 per cent of the total organic solid content of the whole pollen extract.

The effect on the hemagglutination reaction of heating the serum at 56° C. for six hours to destroy the skin-sensitizing activity is shown in Table IV. The serum of six patients was divided into two tubes—one heated, and one kept at room temperature. The hemagglutination titer was not diminished, and may even have been increased by heating.

TABLE IV. THE EFFECT ON THE HEMAGGLUTINATION REACTION OF HEATING SERUM FROM TREATED PATIENTS AT 56° C. FOR 6 HOURS

	HEMAGGLUTINATION TITERS					
	UNHEATED SERUM			HEATED SERUM		
SERUM	WHOLE POLLEN	0-20% SATURATED	49-55% SATURATED	WHOLE POLLEN	0-20% SATURATED	49-55% SATURATED
A. K.	16	64	8	16	256*	4
N. C.	4	64	4	8	256*	8
M. H.	32	4,096*	16	32	4,096*	16
E. F.	8	128	8	4	256*	4
A. R. P.	4	3,072	0	4	4,096*	8
J. S.	16	64	8	4	512	4

*Maximum dilution carried out.

Since the hemagglutination titers of the 0-20 per cent fraction were much higher than those of the whole pollen, we attempted to inhibit the agglutination of the 0-20 per cent fraction sensitized cells by mixing the serum with an excess of the 49-55 per cent fraction. Three sera were tested. One serum was from an untreated timothy-sensitive patient, one from a patient last treated eighteen months previously, and one currently receiving timothy injection treatment. Each serum was diluted 1:4 with 0.9 per cent saline and 0.25 ml. was mixed

with 0.3 mg. total organic solids (a tenfold excess) of the 49-55 per cent fraction and incubated at room temperature for ten minutes. The hemagglutination titers of this mixture were compared with those of the serum alone (Table V). We failed to find evidence of inhibition of the hemagglutination of the 0-20 per cent coupled cells by the 49-55 per cent fraction. This result suggests that the 49-55 per cent fraction does not form a stable complex with the hemagglutinating antibody for the 0-20 per cent fraction.

TABLE V. RESULTS OF INHIBITION STUDY USING TENFOLD EXCESS OF 49-55 PER CENT SATURATED FRACTION BEFORE ADDITION OF 0-20 PER CENT SENSITIZED RED BLOOD CELLS

SERUM	WHOLE POLLEN	0-20% SATURATED	0-20% + 49-55%	49-55% SATURATED
N. McC.	32	512*	512*	64
R. D.	8	512*	512*	32
J. R.	8	512*	512*	8

*Maximum dilution carried out.

In an attempt to clarify the nature of the antibody involved in the hemagglutination reaction we undertook preliminary passive transfer studies with one representative serum from an untreated timothy-sensitive patient.

The reagin neutralization technique was used; 0.1 ml. of serum was diluted 1:40 in Coca's solution and mixed with 0.006 mg., 0.01 mg., 0.03 mg., 0.06 mg., 0.1 mg. of total organic solids of whole pollen, 0-20 per cent fraction, and 49-55 per cent fraction and incubated overnight at 4° C. One-tenth milliliter of each mixture was injected into the skin of volunteers not allergic to timothy pollen, and each site challenged eight to twenty-four hours later with 0.1 mg. total organic solids of whole pollen. Reagin was neutralized by 0.01 mg. of whole pollen and 0.006 mg. of the 49-55 per cent fractions, but not by 0.1 mg. of the 0-20 per cent fraction.

One-half milliliter of this reagin containing serum diluted 1/10 in saline was mixed with 0.1 ml. of a 50 per cent suspension of rabbit red blood cells coupled to the whole pollen, to the 0-20 per cent, and to the 49-55 per cent fractions. The mixtures were incubated overnight at room temperature. The supernatant was tested by the reagin neutralization technique just described. No adsorption of reagin by cells coupled with whole pollen or any fraction was demonstrated.

A serum from a treated patient was heated at 56° C. for six hours, and demonstrated to contain blocking antibody to whole pollen by inhibition of reagin neutralization. One-tenth milliliter of the 1/40 dilution of serum containing reagin used in the preceding experiment was mixed with 0.1 ml. of a 1/40 dilution of the heated serum containing blocking antibody and 0.006 mg., 0.01 mg., 0.03 mg., 0.06 mg., 0.1 mg. of whole pollen, 0-20 per cent and 49-55 per cent fractions. Each mixture was incubated overnight at 4° C. One-tenth milliliter of each mixture was injected into the skin of a normal volunteer and challenged twenty-four hours later with 0.1 mg. whole pollen. Blocking antibody was found to block the neutralization of reagin by the 49-55 per cent fraction

as well as by whole pollen. Again the 0-20 per cent fraction did not neutralize reagin. One-half milliliter of a 1:10 dilution of the serum containing blocking antibody was mixed with 0.05 ml. of packed rabbit red blood cells coupled with WP, 0-20 per cent and 49-55 per cent fractions, and each mixture was incubated overnight at room temperature. The cells were removed by centrifugation and the supernatant tested for blocking antibody activity by the reagin neutralization technique just described. The blocking antibody was adsorbed by the cells coupled with whole pollen and the 49-55 per cent fraction, but not the 0-20 per cent fraction.

DISCUSSION AND CONCLUSION

The results of these experiments indicate that the hemagglutination reaction of rabbit red blood cells coupled to timothy pollen by diazo bonds is a specific and fairly reproducible phenomenon. Assuming, for purposes of discussion, that it is an antigen-antibody reaction, what antibody is involved? The following results indicate that the antibody is not reagin (skin-sensitizing antibody): (1) heating the serum did not diminish the hemagglutination titer; (2) there is a sharp separation of the fraction in timothy pollen producing hemagglutination from the fraction producing both the direct and passive transfer skin reaction; (3) reagin was not absorbed from serum by the red blood cells coupled to pollen; (4) incubation of serum with the 49-55 per cent fraction which has high affinity for reagin in the skin did not inhibit the hemagglutination of cells coupled to the 0-20 per cent fraction.

The hemagglutinating antibody has several of the properties of blocking antibody. It is heat stable, and is present in higher titer in treated than in untreated patients in those few cases we actually tested. However, the results of our passive transfer studies indicate that blocking antibody is not absorbed by red blood cells coupled to the antigen most reactive in the hemagglutination test, but is adsorbed by red blood cells coupled to the skin reactive antigen. Moreover, the technique for the demonstration of blocking antibody defines this antibody as reacting with the skin reactive antigen. So the hemagglutinating antibody to the 0-20 per cent fraction must be distinct from blocking antibody as it is customarily defined, although they share some of the properties of "classical" as contrasted to "reaginic" antibodies. The adsorption of blocking antibody by the cells coupled to WP and the 49-55 per cent fraction is evidence that the pollen allergen is actually attached to the red blood cell, and that antibody binding sites are available to combine with antibody of the same specificity as that involved in the timothy sensitivity of the patients. The finding that the 49-55 per cent fraction is coupled to red blood cells demonstrates that the low hemagglutination titers of the 49-55 per cent fraction is not due to failure of coupling of antigen to red blood cells, but due to failure of reagin to agglutinate these cells.

The failure to demonstrate adsorption of reagin by these cells coupled to whole pollen and the 49-55 per cent fraction, while blocking antibody was adsorbed, suggests that the bond between allergen and blocking antibody is stronger than the bond between allergen and reagin.

If our results can be confirmed and extended in other laboratories, they may indicate that there is an antibody other than reagin and blocking antibody in untreated allergic sera, and that the antigen for this antibody is not the same as that for reagin and blocking antibody. In any case, this test is not suitable for an in vitro assay of reagin or the allergen in timothy pollen responsible for the skin test.

SUMMARY

The hemagglutination of rabbit red blood cells coupled to timothy pollen extract by diazo bonds was explored as in vitro assay for timothy allergen during fractionation and purification procedures. We found that rabbit red blood cells coupled to the fraction of timothy pollen precipitated by 20 per cent saturated ammonium sulfate were agglutinated by sera of patients allergic to timothy pollen, but that this fraction gave very weak skin test reactions. The fraction soluble in 49 per cent but precipitated by 55 per cent saturated ammonium sulfate gave low hemagglutination titers, but strongly positive skin tests. Passive transfer tests indicated that the hemagglutinating factor is distinct from reagin and blocking antibody. The separation of the fraction in timothy pollen producing hemagglutination from the fraction producing the skin reaction indicated that reagin and the antigen in timothy pollen responsible for the skin reaction are not involved in the hemagglutination reaction.

We would like to express our gratitude to Dr. T. E. King, Science Research Institute, Oregon State College, for the many hours of his time given for the discussion of this problem.

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REPOSITORY ANTIGEN INJECTIONS

Absorption Studies by Immunologic and Radioactive Methods

Samuel M. Feinberg, M.D., Haskell I. Rabinowitz, M.D., Jacob J. Pruzansky, Ph.D., Alan R. Feinberg, M.D., and Arthur Kaminker, M.D., Chicago, Ill.

THE INJECTION of a pollen extract emulsified in mineral oil has been used by Loveless[1] and Brown[2] as a repository form of treatment for desensitization. They have referred to the efficacy of this method as being due to a slow absorption of the antigen. Before any new or modified form of therapy is adopted for clinical use, it is the responsibility of physicians concerned to investigate the safety, efficacy, and mechanisms of its action. Several questions arise with respect to the repository treatment: (1) How much of the antigen is in the free aqueous phase ready to produce early local or constitutional reactions? (2) How long and to what extent does the antigen persist at the site? (3) Is the antigen available distant from the site or is it perhaps metabolized in situ? (4) Is there a possibility of inducing immediate or delayed sensitivity? (5) What effect might result from the presence of a foreign body locally? (6) Can objective methods be utilized to show the efficiency or superiority of such treatment over the conventional method?

The investigations to be described here do not purport to give the answers to all these questions, but it is hoped that the data obtained on some phases will help stimulate further investigations.

METHODS AND PROCEDURES

Emulsions were prepared as recommended by Brown, with slight modification. One part of a ragweed pollen extract which had been prepared by extraction with a Coca-Merthiolate solution was mixed with 2 parts of a 10 per cent solution of Arlacel-A in light paraffin oil and emulsified with the aid of two syringes connected with a joint needle. The details will be described in a subsequent article.

Reaction of Passively Sensitized Sites Following Deep Subcutaneous or Intramuscular Injection of Antigen.—Several years ago we had shown[3] that the speed and degree of absorption of an antigen from a subcutaneous or in-

From the Allergy Research Laboratory, Department of Medicine, Northwestern University Medical School.

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tramuscular injection could be determined by the rate and end point of reactions of sites sensitized with serial dilutions of reagins. The skin of the forearms or back was injected intradermally with 0.1 ml. quantities of serum containing ragweed reagins. On the following day deep subcutaneous injections of 1.0 ml. of an aqueous extract of ragweed, containing 10,000 pollen units, were made in the arms of nonallergic subjects. In other subjects, 1.0 ml. of the emulsified preparation, containing the same number of pollen units, was injected. In some instances, after new sites were sensitized the emulsion was injected in the same subject who had had aqueous injections several weeks earlier.

Titration of Aqueous Phase by Direct Scratch Tests on Very Sensitive Allergic Subjects.—Several ragweed-sensitive subjects with varying degrees of skin sensitivity were titrated with serial dilutions of pollen extract. Ragweed pollen extract, containing 50,000 pollen units per milliliter, was prepared in ten numbered threefold serial dilutions so that the range varied from 50,000 units per milliliter in No. 1 to 2.55 units in No. 10. A drop of each solution was placed on a scratch on the forearm. Those subjects suspected of being highly sensitive were not tested with the first two or three strengths. Occasionally even more dilute solutions than the No. 10 were required to reach an end point. The emulsified extract was tested by scratch on the same subject. A positive reaction to the emulsion indicated that it had at least as much (and possibly more) free active antigen as the weakest aqueous extract giving a positive test. Failure of the emulsion to react indicated that the amount of free antigen was less than that contained in the weakest reacting aqueous preparation. The testing of several subjects with varying degrees of reactivity made possible an approximate assay of the emulsion. The following example (Table I) illustrates the method. An emulsion containing 5,000 pollen units

TABLE I. EXAMPLE OF TITRATION OF RAGWEED EMULSION BY COMPARISON WITH SCRATCH TEST REACTIONS TO DILUTIONS OF AQUEOUS EXTRACTS ON ALLERGIC SUBJECTS

DILUTION	POLLEN UNITS/ML.	SUBJECT A	SUBJECT B	SUBJECT C
1. 1:20	50,000	+	+	...
2. 1:60	16,666	+	+	...
3. 1:180	5,555	+	+	...
4. 1:540	1,852	+	+	+
5. 1:1,620	617	+	+	+
6. 1:4,860	206	+	+	+
7. 1:14,580	69	+	+	+
8. 1:43,740	23	0	+	+
9. 1:131,220	7.66	0	0	+
10. 1:393,660	2.55	0	0	+
11. 1:1,180,980	0.85	...	...	+
12. 1:3,542,940	0.28	...	...	0
13. 1:10,628,820	0.09	...	...	0
Emulsion reactions		0	+	+

per milliliter tested on the subjects, whose sensitivities are noted, reacted in subjects B and C but not in subject A. A reaction in subject C indicates that the preparation contained more than 38 units per milliliter. A reaction in subject B indicates that it contained more than 7.66 units. A negative reaction

in subject A indicates that there were less than 69 free units. Thus, it would be interpreted that this emulsion contained between 38 (0.76 per cent) and 69 (1.38 per cent) units in the aqueous phase.

The amount of free antigen could be judged also from the local subcutaneous reaction in allergic subjects. This will be discussed further in the article to follow. By suspending 0.1 ml. of radioiodinated emulsion in 2 ml. of Coca's solution and determining the radioactivity in the surrounding Coca's solution within a very short time, we made an additional estimation of the free aqueous phase.

Neutralization of Passively Sensitized Sites.—The problem here was to note whether the presence in the circulation of antigen, presumed to be released gradually from the emulsion, could be ascertained by its neutralizing effect on passively sensitized sites. The appropriate dilution of reagin was determined in the following manner. Several nonallergic subjects were sensitized with twofold serial dilutions of reagin. A subcutaneous injection of 10,000 pollen units in aqueous extract was made at a remote site. A dilution of reagin which was weaker than that reacting by this method was selected for intradermal injection in a number of sites in the same subject. One row of sites was injected intradermally with 0.02 ml. of threefold serial ragweed dilutions, ranging from 1:1,000 to 1:729,000 and the lowest concentration of pollen giving a reaction was noted. This titration established the base-line reactivity. A subcutaneous injection of 10,000 units of pollen, in either aqueous extract or emulsion, was injected immediately thereafter. Twenty-four hours later another row of sensitized sites was tested with various pollen concentrations in order to note any change in reactivity. In the case of the emulsions, we repeated this procedure at intervals of days, then weeks, always using fresh sites. In order to account for normal inactivation of reagin in the site, control sites were simultaneously sensitized with an unrelated reagin (Alternaria) and subsequently tested with its antigen.

Radioiodinated Ragweed Pollen Extracts.—Preparations of I^{131}-labeled ragweed extract were made by iodination of protein, according to the method of Pressman.[4] As determined by scratch-test titrations on allergic subjects, this procedure did not alter the allergenic activity of the extract. These preparations were injected in emulsion form into three nonatopic and two ragweed-sensitive persons. In aqueous form, a deep subcutaneous injection of the I^{131}-labeled preparation was given to one nonatopic and one ragweed-sensitive person. Two separate batches of this labeled extract were made up. The first, which was given to the three nonatopic subjects who received emulsion and the two subjects who received aqueous antigen, contained approximately 1 microcurie of radioactivity per injection. The second batch, containing approximately 10 microcuries per 1 ml. of emulsion and 3,000 pollen units of antigen, was administered to the two allergic subjects. The fate of the antigen was studied by two methods. In all subjects, radioactivity at the site of injection was counted with a sodium iodide crystal and large 2 or 3

inch aperture lead shield. Counts were made immediately after injection and at intervals thereafter until the activity became impossible to measure, due to either biologic elimination or radioactive decay (half-life of I^{131} = 8.07 days). In the two atopic persons who received labeled emulsion, the radioactivity in the urine and blood was counted in a well scintillation counter. It was not possible to obtain radioactivity counts in the other subjects because of the relatively low radiologic activity of the injected material. The radioactivity eliminated in the urine was determined in one subject for the first six days after injection. Unfortunately, because of the unavailability of the other subject for a period of time after the injection, it was possible to collect the urine specimens only in the first six hours.

In one nonallergic subject, an attempt was made to detect radiation at the site of passive transfer of reaginic serum after injection of I^{131}-labeled emulsion at a distant site. Activity over the regional lymph nodes and thyroid gland was investigated in all subjects who received I^{131}-labeled material.

Induced Sensitivity.—Several nonallergic subjects who had received aqueous or emulsified pollen extract were tested for immediate and delayed skin reactivity with intradermal injections of pollen, varying in strength from 1:100,000 to 1:100. Several allergic subjects who had received the emulsions were also tested for delayed sensitivity.

Assay of Skin, Eye, and Nose Sensitivity.—This will be described in the article to follow.

RESULTS

Amount of Active Antigen in Aqueous Phase Ready for Release.—When deep subcutaneous injections of 10,000 units of aqueous pollen extract were given to a passively sensitized person, the sites sensitized with the reaginic serum, diluted 1:32, were the weakest in which reactions were usually obtained. An emulsified antigen administered later in such subjects or in other subjects sensitized with the same serum failed to show reactions at the sites sensitized, even with the undiluted serum. The direct scratch test with the emulsion indicated that approximately 1 per cent of the preparation was in the active aqueous phase. This estimation was further corroborated by the 1 per cent radioactivity found in the Coca's solution in which a small amount of emulsion was placed. Preparations made by us with very strong pollen extracts and with reduction of the proportion of Arlacel A-oil combination showed a considerably greater percentage of free antigen. The reactivity of the emulsion, as noted by the reaction after the subcutaneous injection in the allergic person, varied. For example, subject A, who had received treatment with 200 units of aqueous extract with moderate reaction, showed no reaction when injected with 2,500 units of the emulsion. Obviously, all that could be ascertained from that trial was that the emulsion was less than 8 per cent active; how much less is impossible to say. Another preparation containing 3,000 units in emulsion evoked a slight reaction in one person

whose reaction to 50 aqueous units was moderate, while in another subject with a moderate reaction to 25 units a marked swelling resulted from the same emulsion. This would indicate perhaps that between 1 and 2 per cent of that emulsion was in the free aqueous phase.

It was found that the radioactivity readings over the site of injection of the emulsion for the first few hours were unreliable.

Persistence of Antigen at Site.—The one allergic person who received emulsion and showed a significant drop (37 per cent) in the first two and one-half hours was the most sensitive of the subjects studied. Both of the ragweed-sensitive persons who received emulsion injections showed a more rapid rate of disappearance of I^{131} from the injection site than did the non-

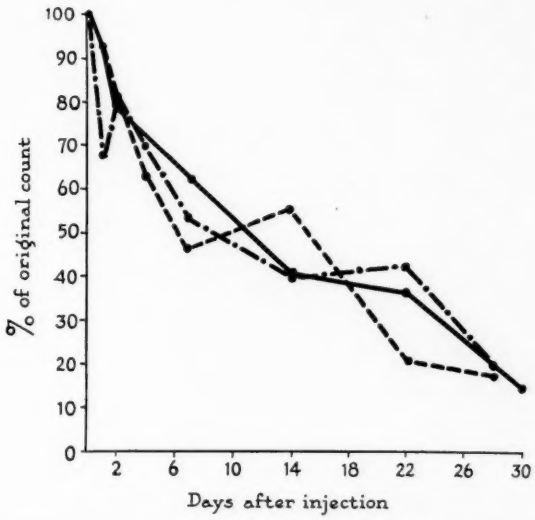

Fig. 1.—Disappearance of radioactivity from sites of injection of 1 ml. of I^{131} ragweed emulsion in three nonallergic subjects. Readings expressed as per cent of original count after correction for radiologic decay of I^{131}.

atopic subjects who received emulsion (Figs. 1 and 2). There was a gradual disappearance of radioactivity from the site until only about 20 per cent (corrected for loss due to radioactive decay) remained after four weeks in the nonatopic group (Fig. 1). Because of the relatively short half-life of I^{131} (8.07 days), this was as long as readings could be obtained in this group of those who had received approximately 1 microcurie of radioactivity in the original injection. In the two allergic subjects who received emulsified pollen extract containing 10 microcuries, it was still possible to show significant radioactivity (corrected to about 5 per cent) at the injection site after eight weeks (Fig. 2).

In the two subjects sensitive to ragweed pollen, radioactivity was removed from the site at a much more rapid rate (Fig. 2). Both of these persons had eliminated approximately 1 per cent of the calculated injected radioactivity in the urine within two hours and a total of 4 per cent in the first eight hours. A total of 31 per cent of the activity appeared in the urine of the one person

TABLE II. NEUTRALIZATION OF SENSITIZED SITES BY 10,000 UNITS OF RAGWEED EXTRACT IN AQUEOUS AND EMULSIFIED FORMS

SUBJECT	PREINJECTION TITER	1 DAY AFTER AQUEOUS INJECTION	PREINJECTION TITER	TITERS AFTER EMULSION INJECTION							
				1 DAY	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	5 WEEKS	6 WEEKS	10 WEEKS
A	1:81,000	1:27,000	1: 81,000	1:27,000		1:27,000	1:27,000				
B	1:81,000	1:27,000	1:243,000	1:81,000	1:27,000	1:27,000		1:27,000			
Alternaria			1: 9,000	1: 9,000	1: 9,000			1: 9,000			
C	1:81,000	1:27,000	1:243,000	1: 9,000	1: 9,000	1: 9,000			1:9,000		
Alternaria			1: 27,000	1:27,000	1:27,000	1:27,000					
D	1:81,000	1:27,000	1: 81,000	1: 9,000	1: 9,000		1: 3,000			1:3,000	1:1,000
Alternaria			1: 27,000	1:27,000	1:27,000		1: 3,000				1:1,000

in whom the excretion of I^{131} in the urine was followed for six days. The chemical combination of I^{131} as it appeared in the urine or blood is not known. It was no longer bound to protein, as it would not precipitate with ammonium sulfate at 50 or 100 per cent saturation, trichloroacetic acid, or heat coagulation. By these methods it was readily precipitable in the original iodinated extract.

The studies of the absorption of I^{131} ragweed extract in aqueous solution showed no difference in the rate between the ragweed-sensitive and the nonatopic persons. As expected, the absorption rate was much more rapid than in those persons who received emulsified preparations. However, over 20 per cent remained after twenty-four hours and between 10 and 15 per cent

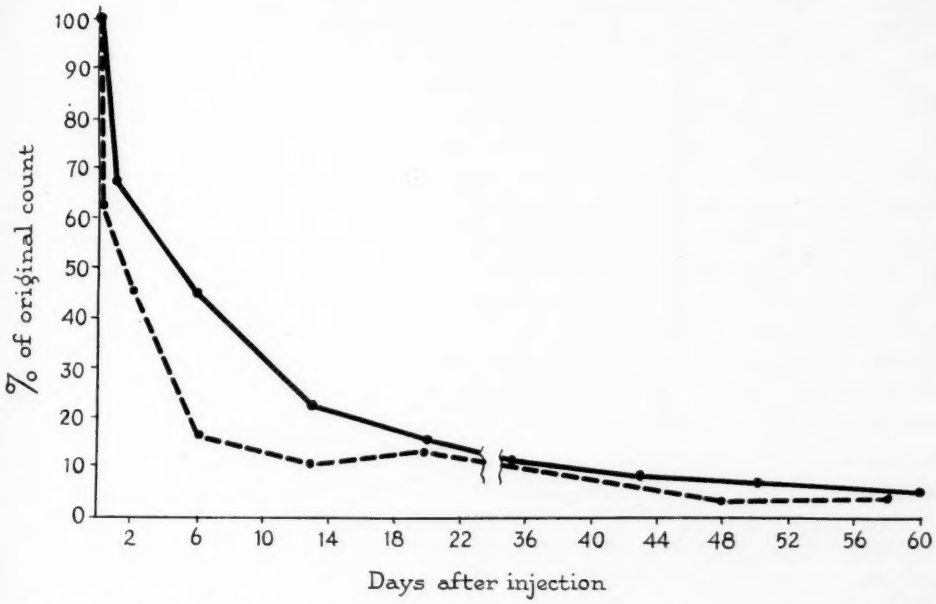

Fig. 2.—Disappearance of radioactivity (corrected) from sites of injection of 1 ml. of I^{131} ragweed emulsion in two allergic subjects.

after forty-eight hours. Between 2 and 4 per cent of the radioactivity originally injected was present at the site as long as three weeks after injection in one subject and two weeks after injection in the other (Fig. 3).

Prolonged Release of Antigen From Emulsion.—The rate of release of antigen from the emulsion and the aqueous extract can be followed from the curves of diminished radioactivity at the site of injection. Whether the radioactivity released actually represents antigen capable of stimulating protection is not known. The experiments on reagin neutralzation might offer a clue. Table II shows the effect of both aqueous and emulsified antigens on the reagin. It will be seen that, within the limitations of the experiment, neutralization cannot be shown to occur consistently after the first twenty-four hours. No radioactivity of remote passively sensitized sites was noted after the I^{131} emulsion injections.

No significant radioactivity could be found over the general body surface or in the regional lymph glands at any time after the injection. Radioactivity over the thyroid gland was considerable in the subjects who had received no iodides prior to the injections and moderate in those who had been given iodides.

Production of Delayed Hypersensitivity.—Of six nonallergic subjects who had received 10,000 units of emulsified ragweed pollen extract and who were tested subsequently, five showed positive delayed reactions to intradermal tests with pollen. These reactions varied from moderate to very strong. In one subject, a 1:1,000 solution injected intradermally produced marked inflammation

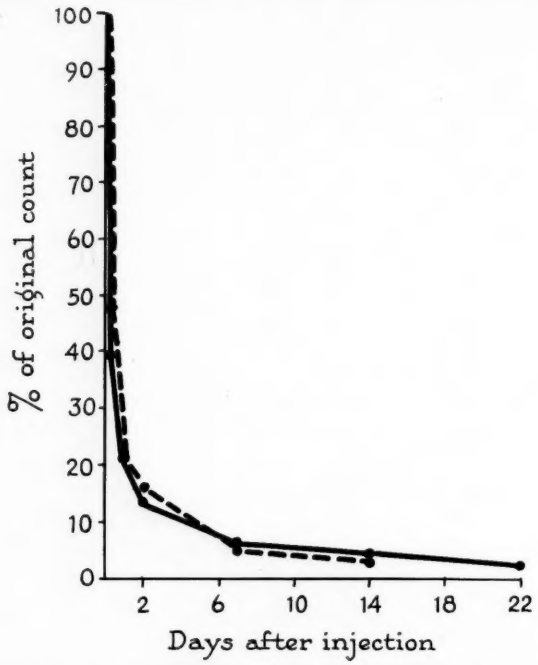

Fig. 3.—Disappearance of radioactivity (corrected) from sites of injection of 1 ml. of aqueous I^{131} ragweed extract in one allergic and one nonallergic subject.

with necrosis. It is not known when this delayed hypersensitivity began, but the first test was made five weeks after injection of the emulsion. In some of the subjects retested as long as three months after the emulsion injections, a positive reaction was still obtainable. Gel diffusion tests for precipitating antibodies to ragweed pollen antigens were negative.

The subject who had the most marked delayed skin reaction began to develop soreness, limitation of movement, and axillary gland enlargement in the arm which had received the emulsion two weeks after the injection. This reaction progressed to marked redness, swelling to about one and one-half to two times its size, then to softening and fluctuations, and finally to spontaneous drainage of sterile pus about three months after the injection. During the height of this inflammation the subject also developed typical lesions of

erythema nodosum. At the time of the development of the erythema nodosum, he informed us that he had had a sore throat a few days before and a cold at the time of the injection of emulsion.

Objective Evidence of Protection From Emulsified Antigens.—This will be discussed in a subsequent article.

DISCUSSION

A more direct way to study the reactivity of passively sensitized sites undoubtedly would have been to determine first the smallest amount of antigen in aqueous medium and then the smallest amount of emulsified antigen required for production of a reaction. We had planned to do this later, since we realized that a large number of subjects would be required. However, the occurrence of delayed reactivity following injection of the emulsified antigen in nonallergic subjects dissuaded us from proceeding along these lines.

The scratch test technique for assaying the reactivity of the emulsion could be made more accurate if there were always available a number of subjects whose differences in reactivity varied by only one dilution of the aqueous extract. This is not easily attainable and, even if it were, only threefold differences in reactivity of the emulsion could be determined. However, the method is satisfactory for rough screening to ascertain whether an emulsion is poor, good, or excellent. The local reaction occurring in the intervening twenty-four hours or so following the administration of the emulsified antigen in a subject whose tolerance is known gives an approximate indication of the amount of readily releasable antigen. There is a marked difference in local reactivity, however, dependent on unrecognized variations in the administration of the antigen and perhaps fortuitous spread of the material close to the skin surface. An example will illustrate the point. One of us injected 0.5 ml. of an emulsion of 5,000 units per milliliter into one arm and an equal volume from the same syringe into the other arm. One arm had no reaction whatsoever. The other arm began to itch shortly after the injection, and by the end of twenty-four hours an erythema and swelling had extended over practically the entire arm.

The diminution of radioactivity at the site of injection of emulsion does not necessarily prove that the difference is a measure of release of active pollen antigen. The radioactivity loss during the first twenty-four hours varied markedly in different persons. Perhaps this may be partly explained by the spread of the material to different depths in the tissues. Horizontal spread of material was measured by radiographic scanning as is employed in thyroid studies (Fig. 4). Here it will be seen that there are variations in subjects or perhaps slight differences in the technique of injection. It is also possible that the pollen antigen may be metabolized to a considerable extent at the site of injection and thus not be available as an active antigen in blood and distant tissues. The possibility of such an occurrence is supported to some extent by the failure of neutralization of sensitized sites to proceed beyond a twenty-four-hour period. It may be that this is due to the fact that either the antigen is metabolized in situ or that small amounts released in active form are still

capable of being inactivated enzymatically before combining with antibody. The finding that a preponderance of radioactivity in the blood is in uncombined form also suggests that the antigen is rapidly metabolized.

It will also be noted that the rate of loss of radioactivity appears to be in the following order of increasing rapidity: (1) from emulsions in nonallergic subjects, (2) from emulsions in allergic subjects, and (3) from aqueous extracts. This, too, would suggest a more rapid metabolism of the antigen in situ by the allergic person. The apparent, more rapid diminution of radioactivity at the injection sites in allergic subjects who had received the emulsified antigen may be accounted for by the reaction of the tissues to the free aqueous antigen.

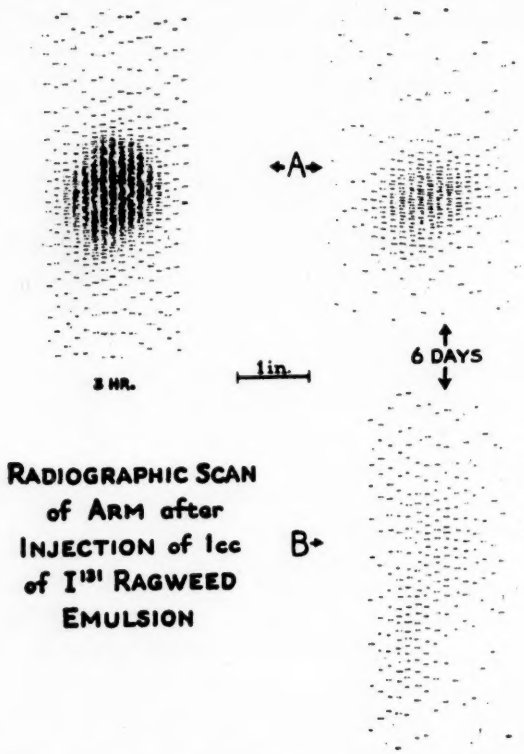

Fig. 4.—Pattern of spread of radioactivity in arms of two allergic subjects after injection of 1 ml. of I^{131} ragweed emulsion. Scannings obtained with a Model 1700 isotope scanner made by Nuclear Chicago. Each small bar represents thirty-two detected disintegrations.

The resulting increased vascular permeability, possibly increased phagocyte infiltration, enzymatic mechanism dependent on the antigen-antibody reaction, or other inflammatory changes may be responsible. The finding of measurable radioactivity in situ from injections of aqueous extracts for as long as three weeks is rather surprising in view of previous concepts about absorption of aqueous extracts. In a similar study in rabbits with radioiodinated bovine gamma globulin and egg albumin, Talmage and Dixon[5] in 1953, reported the disappearance rate from the emulsion to be similar to that which we found in

man. With the aqueous preparation, however, only 1 per cent of the bovine gamma globulin persisted at the site after two days, and less than 1 per cent of the egg albumin remained after one day. Is our finding true for ragweed protein and not for other proteins? Is it likely to be shown with injected uncombined I^{131}? All these points require further investigation. Nevertheless, this finding does cast some doubt on the validity of the concept that it is only the persistence of antigen which accounts for the superiority of the repository treatment, since some antigen does persist even with the aqueous extracts. It should be realized also that the natural decay of I^{131} places practical limitations on the ability to locate remnants of the antigen for a long time with either the aqueous or the repository preparations. In eight weeks an initial count of 10,000 will be reduced to about 40, a count difficult to detect.

It should be emphasized here that because of the very nature of the difficulty of conducting all experiments with the same batch of emulsion the emulsions may have varied from time to time. This may account for the difference in quantities estimated to be released as determined by different methods. It should be noted at this point also that we may be far from correct in discussing radioactivity as if it were synonymous with antigen. It may be that only part of the radioiodinated pollen extract is actually the antigenic material. The separation of antigenic from nonantigenic components is of course a problem of issue in this and other fields of investigation. We are proceeding with attempts at such separation.

The production of delayed hypersensitivity poses both basic and practical questions. If the emulsion stimulates the immune response, it may produce several effects. It might induce delayed hypersensitivity to the injected antigen, as it has done in several of these subjects. It might produce sensitivity to another antigen, as to microbes present in the body at the time of injection. There is a strong suggestion of this possibility in the subject who developed erythema nodosum. This delayed reactivity appears to have occurred only in nonallergic subjects. However, more extensive investigations are planned to corroborate this phenomenon and to attempt to clarify it.

SUMMARY

1. Methods employed to study the degree of immediate release of ragweed pollen antigen from emulsions have consisted of the ability of emulsified and injected antigens to produce reaction in passively sensitized sites, the reaction on scratch test in allergic subjects after titration with aqueous extracts, the radioactivity of the blood and urine shortly after the injection of radioiodinated emulsion, the local reaction of the emulsion in allergic persons, and the radioactivity release from the emulsion placed in an aqueous solution.

2. With the emulsions studied, such immediate release varies from about 1 to 3 per cent, depending on the method used and the batch of the emulsion.

3. Persistence of the antigen at the site of injection has been studied in relation to the persistence of radioactivity of radioiodinated pollen extract. Traces of the radioiodination can be shown to exist for as long as four weeks

with the emulsion and three weeks with the aqueous preparation. It is possible that longer persistence might be shown with our use of more potent radioactivity.

4. By the method of reagin neutralization, it could not be proved that antigen is released from the emulsion in active form beyond the twenty-four-hour period. It is admitted that this is not final evidence that the antigen is unavailable.

5. The production of delayed hypersensitivity with the emulsified antigen in nonallergic subjects is of basic and clinical significance.

6. Further amplification of these and other studies is needed to perfect the antigenic preparation and to understand the immune mechanisms involved.

We are grateful to Dr. R. Dowben of the Department of Medicine, Dr. W. Moss of the Department of Radiology, and Dr. R. Shaw of the Department of Biochemistry for their valuable assistance and advice in the use of radioisotopes in this study.

ADDENDUM

In five additional nonallergic persons, we have been able to confirm the persistence of some radioactivity at the site of injection of aqueous ragweed extracts for periods up to three weeks.

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REPOSITORY ANTIGEN INJECTIONS

Preparation and Therapy

Alan R. Feinberg, M.D., Samuel M. Feinberg, M.D., and Elmer W. Fisherman, M.D., Chicago, Ill.

THE USE of an allergen emulsified in oil for the treatment of hay fever and asthma caused by pollens has recently been advocated by Loveless[1] and Brown.[2, 3] The latter has claimed[4, 5] that 82 to 88.5 per cent of the patients thus treated have gone through pollen seasons without a single sneeze. These results, together with the other advantages to be obtained with a drastic reduction in the number of injections necessary, have stimulated us to look further into the possibilities of this form of therapy.

BACKGROUND

Although the conventional form of desensitization treatment has long been recognized as effective, many aspects of it are in need of improvement. Enhancing therapeutic results, saving of time and expense with fewer injections, and increasing the safety would be highly advantageous steps. It has been claimed that the repository treatment, in which an allergen-in-oil emulsion is used, offers these advantages. The purpose of this investigation is to try to determine whether or not these objectives are met. In another article we have presented some experimental aspects of this subject.[5a]

For many years, attempts have been made to slow the absorption of allergen with the hope of being able to diminish the number of injections and increase their safety. Early workers added extracted or unextracted pollen to vegetable oil; later, better emulsions in vegetable oil were made and absorption could be definitely slowed.[6] However, primarily because of resulting local reactions consisting of unabsorbed nodules, fibrosis, and suppuration, these procedures were usually abandoned. The ragweed allergen itself has been altered by chemical changes, as with the use of tannate[7] or alum-precipitated allergen,[8] but the desensitation required multiple injections and results were not superior.

Since 1931, in our laboratory, various preparations have been made in an attempt to achieve the previously mentioned objectives. These have included

From the Allergy Research Laboratory and Allergy Clinic, Department of Medicine, Northwestern University Medical School.

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precipitated pollen protein suspended in oil, whole ground pollen suspended in oil or saline, and oil-in-water and water-in-oil emulsions. With gum dammar as the emulsifying agent for a water-in-vegetable oil emulsion, fairly good results were obtained, but the amount of antigen that could be injected safely was limited and the therapeutic results were not outstanding. The successful use of aluminum monostearate as an emulsifying agent for prolonging the absorption of penicillin suggested its use in the same way with ragweed extract. Sesame oil was used as the oil phase.[9] Therapeutic results were not as good as with conventional treatment.

Freund and co-workers,[10] using paraffin oil as the oil in the water-in-oil emulsion which contained killed Mycobacterium, demonstrated that this acted as a synergistic agent to various antigens in the production of antibody. This was called a complete adjuvant; when the Mycobacterium was omitted, it was called an incomplete adjuvant. Freund and his colleagues found that the incomplete adjuvant would raise the anaphylactic (immediate) type of sensitivity which depends on titer of antibodies, while the complete adjuvant was necessary to produce the delayed type of sensitivity. For his more recent experiments,[11] Freund has used the emulsifying agent Arlacel-A* (mannide mono-oleate), 1.5 parts, to 8.5 parts by volume of any paraffin oil of low specific gravity and low viscosity, such as Bayol F. Equal volumes of the aqueous solution suspension of antigen were used to make the incomplete adjuvant. He found that vegetable oils were ineffective in the adjuvant. In the treatment of hay fever, others had used vegetable oils because they were considered to be less foreign to the body and therefore better tolerated.

In 1947 Loveless,[12] using Freund's work as a basis, reported on her results in the treatment of thirty-three ragweed hay fever patients with an emulsion of Falba, mineral oil, and ragweed extract. In fifty-four injections of the pollen extract with adjuvant, there were nine constitutional reactions, mostly mild. In later studies there were better results and fewer reactions.

In 1950, Salk reported[13] on the use of Arlacel-A, instead of Falba, and Bayol F, instead of a heavier mineral oil used earlier, as the adjuvant in influenza vaccination.[14, 15] Later he found[16] that if the Arlacel-A was specially purified he was able to avoid local reactions. He also changed to Drakeol 6 mineral oil.† By 1953,[17] more than 12,000 injections of influenza vaccine in this form had been given under Salk's direction without evidence of an untoward local reaction.

Freund and Thomson[18] emulsified their mixture by repeatedly withdrawing and injecting with a syringe through a 17 or 18 gauge hypodermic needle placed in a rubber stopper on a bottle containing the ingredients. Berlin and McKinney,[19] in 1958, described an apparatus that consists of two syringes connected by a double-hubbed needle formed by wedging a No. 20 inside a No. 18 needle so that the mixture passes from one syringe to the other through this needle and emulsifies in the process. Brown[2] described a similar apparatus

*Furnished by Atlas Powder Co., Wilmington, Delaware.
†Obtained from Pennsylvania Refining Co., Butler, Pennsylvania.

in 1959. He also emphasized the importance of obtaining an emulsion that is homogeneous and that has as little free aqueous phase as possible. He claims[3] that with his present technique the extract is 99.999 per cent emulsified, but he does not describe how this is determined.

Because of the enthusiastic claims that Loveless and, more particularly, Brown have made for this emulsion-injection method of treatment, we again became interested and decided to investigate this method in its clinical and basic aspects. Through the kindness of Dr. Brown, one of us visited his clinic and observed the preparation and injection of the repository materials. Proceeding cautiously, we tested and gave emulsion injections to twenty-six ragweed-sensitive patients during 1959. The clinical studies on these patients form the basis of the present report.

PREPARATION OF MATERIALS

We have used the methods of Salk,[16] Brown[2, 3] and Berlin,[20] with slight modifications. One part Arlacel-A, specially treated, was added to 9 parts of Drakeol 6-VR white mineral oil, and 2.67 ml. of this mixture was placed in a threaded-neck 75 by 16 mm. test tube, closed by a Teflon-lined screw cap (Kimble No. 45066A), and autoclaved. Seitz filtration also appears to be satisfactory. To this sterile mixture was added 1.33 ml. of the allergenic extract.

The allergenic extract used in these experiments usually consisted of equal parts of short and giant ragweed pollen which was ether defatted and extracted with Coca's solution modified by replacing the phenol with Merthiolate to make a 1:10,000 Merthiolate concentration. This same Coca's solution was used for dilutions. It is important that no glycerin, alcohol, or detergent be present in the extract, as they make the emulsion unstable. The extract is Seitz-filtered and tested for sterility. To the dilutions of extract to be used for treatment may be added 0.05 ml. of a 0.05 per cent Evans blue dye solution per 1.33 ml. (a 1:10 dilution of Evans blue dye as prepared for blood-volume studies by Warner-Chilcott Laboratories, Morris Plains, New Jersey) to be used as a gross and microscopic indication of the aqueous phase to help determine completeness of emulsification and emulsion stability. The extract is then added with sterile precautions to the test tube containing the oil mixture. This may be stored in the refrigerator and, as the aqueous phase is not exposed to air because it is covered by oil, it should retain its potency well.

A number of hours before the time of treatment the emulsion is prepared and tested. The test tube containing the oil, Arlacel-A, and ragweed extract is tapped on the side so as to make a crude emulsion. Then this mixture is drawn into a sterile 10 ml. Luer-Lok syringe (those that are tight fitting and have thick walls are best) and through a No. 15 needle, 3 to 4 inches long with the end ground down so that there is no point. The round bottom of the test tube and the shape of the end of the needle then allow all but a very small amount of the mixture to be removed from the tube. The syringe is

held with the needle up, and air is drawn in to remove the mixture from the needle. A sterile No. 18 or No. 19 needle, 1½ to 2 inches long, with a Luer-Lok hub at both ends is attached to the syringe and the air is pushed out. To the other end is attached another 10 ml. syringe. Further manipulation is usually necessary to remove air from the system. The mixture is then pushed back and forth through the needle. As the mixture becomes emulsified more completely, the viscosity increases to the point where it may be difficult to push it through with thumb pressure, and it is necessary to push the plunger against a firm surface to allow the mixture to go through the needle. A small drop is then placed on a glass slide, covered with a cover slip, and examined under the microscope at a magnification of ×400 for homogenicity and particle size. The particles should be very small and uniform in size and shape. A very poor preparation will be seen to separate in the syringe in a few minutes. A preparation that looks satisfactory initially but separates on standing a number of hours should not be used, even if re-emulsified.

The emulsions were tested for amount of free aqueous allergen by scratch testing on subjects with varying degrees of sensitivity and comparing with serial dilutions of the same allergen. By this method we could estimate the maximum amount of free allergen available for immediate absorption. For example, if the weakest-reacting dilution of an aqueous allergen contained 150 units per milliliter and an emulsion containing 10,000 units per milliliter did not react, we then knew that there was less than 150 units of free allergen in the emulsion available for immediate reaction. If the patient who is being treated can be expected to tolerate 150 units of the aqueous extract, it should be safe to inject that emulsion.

Seventy per cent ethyl alcohol was used as an antiseptic at the site of injection, and the skin was dried with sterile cotton after its use. For injection, the emulsion is pushed into one of the 10 ml. syringes; the other syringe is removed and replaced by a 1 ml. tuberculin syringe which is then filled by forcing the emulsion into it. The 1 ml. syringe is removed and a ⅝ inch disposable needle (No. 25) is attached. A deep subcutaneous injection is then made on the upper outer arms, 0.5 ml. in each arm at fifteen- to thirty-minute intervals. Dividing the total volume into two sites produces less local reaction.

High-speed centrifugation is another method of examining the stability and completeness of emulsion. Some separation of the oil and the emulsified particles is to be expected, but there should be no water phase at the bottom of the tube. Still another way of testing is to measure the electrical resistance of the emulsion or its conductivity. The emulsion may be placed in a tuberculin syringe with a metal plunger, such as the asbestos-wound Cooke syringe. A metal needle is placed on the end, and the resistance between the plunger and the needle is measured with an ohmmeter or resistance bridge capable of reading up to 1,000 megohms or more. The resistance of 1 ml. of a good emulsion should be 500 to 1,000 megohms or greater. When the same syringe is used for injection, each dose may be tested just before use for further assurance of the completeness of emulsification. We would not recommend the use of this procedure without a microscopic examination also being done.

PREPARATION AND TREATMENT OF PATIENTS

The patients selected had an unequivocal history of ragweed pollinosis, and most of them had not been treated at all or had not been treated recently with pollen extracts. We tried to select patients who were allergic only to ragweed, or at least who were not allergic to such substances as molds or dust which can produce symptoms at the same time as the ragweed. All were sensitive by scratch testing to ragweed extracts. Dilutions of mixed ragweed extract were prepared, ranging from 50,000 pollen units to 2.5 pollen units in threefold dilutions. The skin was tested with the ten dilutions. In testing the eyes and nose, we began with the weakest solution and increased the concentration at ten- to fifteen-minute intervals until a reaction was produced. One drop was placed in the same eye at each time. A DeVilbiss No. 31 plastic atomizer was pretested for volume dispensed, and seven sprays were administered in the nostril opposite the eye tested. The end result was either a sneeze or definite itching and stuffiness. Tests just described were performed before treatment (preferably not on the same day as the emulsion injection), again just before the ragweed season, and then after the ragweed season.

Patients were observed for about an hour after the injections. It should be noted that no epinephrine was used in the emulsion or administered before, with, or after injection. No antihistamine or other medication was used before the injections; after the injections we supplied antihistamines, which were to be used only in case of reaction. We purposely avoided the use of auxiliary medication so that we could determine the true rate of reaction. The number of patients treated was small. This was partly because we regarded this as a pilot study and partly because we wished to devote our time to more thorough observations of the group.

All the patients were treated during the month of July. The amounts used for treatment are expressed in pollen units. Two patients had only 500 units, two had 5,000 units, two had 1,000 units followed by 5,000 a couple of weeks later, another patient had 1,000 units followed by 2,500 units later, and all the others had 1,000 units.

RESULTS

The results of treatment with the repository ragweed pollen in the twenty-six hay fever patients were as follows: excellent, 4; good, 13; fair, 2; poor, 5; no follow-up, 2. There was no consistent pattern in the results in relation to the severity of the original symptoms, sensitivity tests, or the dose administered in the emulsion. It should be pointed out, however, that the dose administered was not uniform and was determined in large part by the patient's sensitivity. The results are based on the patients' own evaluation of their symptoms and their need for additional medication.

Although there was variation between patients in the relative sensitivity between eye, nose, and skin tests, the sensitivities were high with all tests. Most of the patients reacted to dilutions of 206 to 23 pollen units per milliliter, while an occasional one reacted to a dilution as high as 2.5 per milliliter

(the usual equivalent is about 2 pollen units to 1 protein nitrogen unit). Following repository treatment with pollen, the tests revealed a decreased sensitivity or no change in the majority of patients. However, several patients showed increased sensitivity by these tests. The observed results of the therapy showed poor correlation with the sensitivity tests.

One patient who received 1,000 pollen units had an urticarial reaction about two hours later which was readily relieved with one dose of an antihistamine. This man was given the injection on the same day that he had the sensitivity tests. He was extremely sensitive, and it is possible that absorption from the skin-test sites and nose and eye tests may have contributed to the total available allergen. Several patients experienced local reactions consisting of swelling, tenderness, redness, and itching, which began shortly after the injection and continued at times for several days thereafter. At times there was a marked difference in the local reaction between the two arms, each given the same dose from the same syringe by the same physician. It is very likely that slight differences in depth and location of the material influence the local reactivity.

DISCUSSION

Our results with repository therapy in a small series of ragweed-sensitive patients do not approach in effectiveness those claimed by Brown. This may be due to our use of conservative doses which, however, appeared to be necessary in the very sensitive patients we treated. A large percentage of Brown's patients (44 per cent) failed to react on eye test to a concentration of ragweed pollen 200 times greater than that which caused reaction in the majority of our patients. Our finding may be due also to the fact that the much greater exposure to ragweed pollen in the Midwest prevents better results. The ragweed pollen count in Boston is about that of Northern Wisconsin and Michigan resorts. These factors may account largely for the differences in therapeutic results.

It had been hoped that the effect of the repository treatment on the quantitative reactivity of the shock organ might offer a way of determining its therapeutic effectiveness objectively. The inconsistent results so far obtained with this assay have not made that possible; perhaps an improvement in the method might eventually make it more useful. We are left, therefore, with the problem of subjective interpretation. Unless the results are very dramatic and unusual, some kind of a controlled study, preferably a double-blind approach, would be required to solve the efficacy of repository therapy. The present emulsion appears to release a significant percentage of active antigen. It is necessary to determine the tolerance of each patient and to decide whether or not the amount of free antigen exceeds that tolerance. With such precautions, severe reactions, local or systemic, can be minimized. One cannot, however, arbitrarily administer a definite number of units, say 5,000, and have 50 units quickly released when we know (as in a patient we have recently treated) that systemic reactions occur from 1 unit of pollen. It is recommended that, before the emulsion is administered to a new patient, the patient's tolerance be determined by one or more injections of aqueous extract.

If the repository treatment does prove effective, the problem of treatment of multiple sensitivity presents itself. It appears that this type of therapy must be timed to precede the season by several weeks or longer. Therefore, injections with mold, tree, grass, and ragweed pollen would have to be given at different times. It is likely also that immunity does not last an indefinite number of months. It is very likely that injections for types of sensitivity which last a long time, as in the case of molds or house dust, would have to be repeated several times during the year. One faces the predicament, therefore, of having to give a number of repository injections in such cases. In that event, the advantages of emulsion therapy may not be as great as originally supposed. The cumulative effect of injecting a foreign body each year or several times a year may also be contemplated as a possible hazard.

If corroborated by further studies, our finding (reported in the preceding article) of the production of delayed hypersensitivity in nonallergic subjects who had been injected with an antigen in repository form would make it hazardous to give such treatment to a patient unless a true sensitivity to that antigen had been established. Because of false irritative reactions, injections of mold "mixtures" containing molds to which the patient is not allergic or the injection of dust extract in a patient whose dust sensitivity is questionable might actually be harmful. It is possible that more perfect emulsions will be developed. It is also possible that the slow release of the antigen is not the important issue. It might be, for example, that the insoluble material acts as a stimulant to the reticulo-endothelial system.

Some of the aspects of this problem have been reported in the preceding article. Much more needs to be done to study basically the absorption of the antigen, its fate, its effect on local tissues, the real effectiveness of the method in giving protection to the patient, and other features concerned with safety. It is hoped that this type of treatment will be considered an experimental project for some time until some of the basic questions can be answered satisfactorily.

SUMMARY

1. Using 2 parts of a solution of 10 per cent Arlacel-A in light mineral oil to 1 part of pollen extract in a Coca-Merthiolate medium, we have prepared emulsions which can be given in amounts of 50 to 100 times the aqueous dose tolerated by the patient.

2. The emulsions are examined grossly and microscopically, and the aqueous phase is determined by scratch tests on several subjects who have been tested with serial dilutions of the aqueous preparation of the antigen.

3. The treatment of a small group of ragweed-sensitive patients with such emulsions in the Chicago area has produced encouraging but not superior results. It would appear that protection of ragweed patients is considerably more difficult in areas of greater pollen prevalence than in the Eastern areas.

4. It is possible that with better timing, larger doses, or an improvement in the preparation, the results might be better in the future.

5. The method is not fool-proof. It requires experience in the preparation and examination of the product and in the evaluation of the patient's tolerance. There is also the possibility of local tissue damage and the production of hypersensitivity of the delayed type.

6. The method offers a possible approach in therapy, perhaps in selected cases. It is not yet a method to be adopted for wide, indiscriminate use.

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RELATIONSHIP OF COMPLEMENT TO ALLERGIC HISTAMINE RELEASE IN BLOOD OF RAGWEED-SENSITIVE SUBJECTS

Elliott Middleton, Jr., M.D., and William B. Sherman, M.D., New York, N. Y.
With the technical assistance of Maja Rudin

RECENTLY there has been considerable interest in the relationship of complement (C′) to experimental allergic reactions. That C′ may participate in some way is suggested by the following observations: (1) there is a fall in serum C′ levels in in vivo immune reactions[1, 2]; (2) hemolytic activity of serum declines in experimentally provoked systemic anaphylaxis[3, 4]; (3) in certain disease processes with a presumptive allergic etiology serum C′ is depressed[5-8]; and (4) a serum constituent resembling C′ has been found to participate in passive cutaneous anaphylaxis (or at least in its potentiation by this material).[9, 10] In studies of histamine release from platelets of rabbit blood, Humphrey and Jacques[11] showed that a heat-labile plasma constituent, in addition to antibody and ionic calcium, was required for release to occur. Becker[12] has elaborated on the role of C′ in immune hemolysis, considering this an in vitro model of the allergic reaction, and from it he has constructed a generalization on the participation of C′ in allergic reactions. Mongar and Schild,[13] however, have produced evidence that C′ is not involved in the anaphylactic release of histamine from sensitized guinea pig lung, although calcium ions are necessary.

Earlier work from this laboratory[14, 15] showed that histamine release from leukocytes of allergic subjects will not proceed in the absence of ionic calcium (and possibly magnesium). The relationship of calcium and magnesium to C′ activity suggested that these ions might be involved. Accordingly, the present experiments were performed. The results are consistent with the view that hemolytically active C′ is not involved in histamine release in allergic human beings.

EXPERIMENTAL METHODS

Blood donors for these experiments were persons with clinically significant ragweed hay fever and strongly positive intradermal skin-test reactions to ragweed extract which contained 100 protein nitrogen units per milliliter. A series of 40 ml. aliquots of blood were drawn into siliconized centrifuge

From the Department of Medicine, College of Physicians and Surgeons, Columbia University.
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tubes containing 50 to 100 units of heparin. The cells were packed by centrifugation, 16 to 18 ml. of supernatant plasma (constant from tube to tube) was aspirated from each tube into a separate tube, and the cells then were washed two or three times at room temperature with heparinized Locke-Ringer solution (pH 7.4 to 7.8) which contained 0.5 per cent gelatin to help stabilize the leukocytes during centrifugation. The separated plasma aliquots were then handled in pairs as follows: first pair, untreated control; second pair, heated at 56° C. for thirty minutes (destruction of C′ 1, 2); third pair, treated with zymosan,[10] 20 mg. per milliliter of plasma (removal of C′ 3); fourth pair, treated with ammonia[16] (destruction of C′ 4); fifth pair, treated with phloridzin by dissolving the compound directly in the plasma to a final concentration of 5.0 to 6.3 by 10^{-3}M (interferes with C′ 3 activity[17] in rat and guinea pig serum). Not all pairs were run in each experiment. Before reconstitution with washed cells, all plasmas were kept stoppered at 37° C. and were diluted with 0.15M sodium chloride to the same final extent as was necessary for the ammonia-treated plasmas following neutralization with hydrochloric acid.[16] The over-all dilution was 1:1.5.

The treated plasmas were reconstituted with their cells to a total volume of 39 ml., care being taken to procure an even resuspension of the leukocytes. After equilibration for twenty to thirty minutes at 37° C., 1 ml. of saline solution was added to one tube of each pair and 1 ml. of ragweed extract to the other. The final concentration of ragweed protein N was 50 μg per liter of whole blood. The mixtures were incubated for thirty minutes at 37° C. with occasional mixing and centrifuged, and plasma supernatants were analyzed for histamine[14] and C′.[18] For the latter, 1 ml. aliquots were usually frozen and assayed on another day. The C′ determinations are reported in Table I as 50 per cent hemolytic units (C′ H50), and the figures are corrected for dilution.

RESULTS

It is evident (Table I) that procedures which abolish the hemolytic activity of plasma C′ have little or no effect on the histamine-release reaction, which proceeded 73 to 100 per cent as effectively as the control. Included in Table I are data from several early experiments on the effect of heating plasma (cells washed only two times, C′ determinations not done). From these and the other experiments with thrice-washed cells, it is evident that heating plasma exerts no significant effect on histamine release. Similar results can be seen in the experiments with zymosan, ammonia, and phloridzin.

Table II shows the results of plasma C′ determinations in five experiments which compared the levels after addition of saline solution or ragweed extract. No difference in C′ could be detected, although histamine release occurred as usual. In these experiments the cells were not washed.

As further evidence of the lack of relationship between C′ and histamine release, advantage was taken of the fact that iodoacetate interferes with histamine release.[15] Accordingly, several release reactions were run with and without

TABLE I. RELATIONSHIP OF HEMOLYTIC COMPLEMENT ACTIVITY OF PLASMA TO ALLERGIC HISTAMINE RELEASE FROM WASHED BLOOD CELLS

SUBJECT	TREATMENT OF PLASMA	TREATMENT OF CELLS	C′ H50	HISTAMINE RELEASED (μg/L. PLASMA)	PER CENT OF CONTROL
E. F.	None (control)	Washed 3×	31	56	100
	Heated 56° C., 30 min.	Washed 3×	0	54	96
	Zymosan, 20 mg./ml.	Washed 3×	0	48	86
	Ammonia	Washed 3×	0	42	75
	Phloridzin 6.3 × 10^{-3}M	Washed 3×	30	41	73
D. B.	None (control)	Washed 3×	42	14	100
	Heated 56° C., 30 min.	Washed 3×	0	22	157
	Zymosan, 20 mg./ml.	Washed 3×	0	15	107
	Ammonia	Washed 3×	0	14	100
	None (control)	Washed 3×	32	39	100
	Phloridzin 5 × 10^{-3}M	Washed 3×	28	38	97
F. C.	None (control)	Washed 3×	36	36	100
	Heated 56° C., 30 min.	Washed 3×	0	40	111
	Ammonia	Washed 3×	0	30	83
	Phloridzin 5.2 × 10^{-3}M	Washed 3×	35	32	89
G. G.	None (control)	Washed 2×	--	45	100
	Heated 56° C., 30 min.	Washed 2×	--	43	96
C. B.	None (control)	Washed 2×	--	28	100
	Heated 56° C., 30 min.	Washed 2×	--	28	100
	None (control)	Washed 2×	--	34	100
	Heated 56° C., 30 min.	Washed 2×	--	29	85
	Heated 56° C., 90 min.	Washed 2×	--	26	76

TABLE II. COMPARISON OF PLASMA COMPLEMENT WITH AND WITHOUT ALLERGIC HISTAMINE RELEASE

SUBJECT	C′ H50 AFTER ADDITION OF SALINE SOLUTION	C′ H50 AFTER ADDITION OF RAGWEED*	HISTAMINE RELEASE (μG/L. PLASMA)
Bel.	31	32	42
Set.	35	35	94
	33.5	32	65
Mar.	28	27	26
Lon.	29	28	20

*Final concentration of ragweed was 50 μg protein N per liter whole blood.

TABLE III. RELATIONSHIP OF IODOACETATE INHIBITION OF HISTAMINE RELEASE AND COMPLEMENT

EXPERIMENT	HISTAMINE RELEASED (μG/L. PLASMA) CONTROL	IODOACETATE 10^{-3}M*	C′ H50
1. L. S.	65		34
		10	34
2. A. M.	57		29
		15	27
3. F. L.	20		28
		11	27
4. B. B.	42		31
		11	31
5. L. S.	94		35
		8	33

*Final concentration of iodoacetate in whole blood was 10^{-3}M.

added iodoacetate (10^{-3}M in whole blood). It can be seen in Table III that marked reduction of histamine release in the presence of 10^{-3}M iodoacetate is not accompanied by any change in C′ activity.

To determine the extent to which antibody and/or C′ may be bound to cells, the following experiments were performed. Cells which had been separated and washed three times in heparinized Locke-Ringer solution were resuspended in this medium and antigen was added. Release from these cells was compared with histamine release from similarly treated cells resuspended in their own plasma. The results, shown in Table IV, demonstrated that cells washed

TABLE IV. ALLERGIC HISTAMINE RELEASE FROM BLOOD CELLS WASHED THREE TIMES AND RESUSPENDED IN PLASMA OR LOCKE-RINGER SOLUTION

EXPERIMENT	RAGWEED CONCENTRATION*	HISTAMINE RELEASED (μG/L.) FROM CELLS RESUSPENDED IN		PER CENT OF CONTROL $\frac{b}{a} \times 100$
		PLASMA (a)	LOCKE-RINGER (b)	
1. R. C.	50	46	36	78
2. T. D.	50	79	53	67
	20	89	65	73
	10	62	45	73
3. R. C.	50	41	24	59
	20	52	29	56
	10	52	29	56
4. B. B.	50	50	42	84

*Micrograms of protein N per liter whole blood.

free of plasma release 56 to 84 per cent as much histamine (50 μg ragweed protein N per liter of cell suspension) as washed cells resuspended in their own plasma. In the course of these experiments, it was determined on three occasions that there is essentially no loss of cellular histamine after washing three times (Table V).

TABLE V. EFFECT ON CELLULAR HISTAMINE CONCENTRATION OF WASHING THREE TIMES WITH HEPARINIZED LOCKE-RINGER SOLUTION CONTAINING 0.5 PER CENT GELATIN*

EXPERIMENT	CONCENTRATION OF CELLULAR HISTAMINE (μG/L.)	
	BEFORE WASHING	AFTER WASHING
1	89	105
2	155	132
3	74	77

*Cells were obtained from 40 ml. of whole blood and were finally resuspended in Locke-Ringer solution to the same volume.

If C′ activity is required for histamine release and if washing leukocytes does not remove C′ which might be bound, then C′ activity might be found in extracts of washed leukocytes. In several experiments, leukocytes (admixed with about 50 per cent red cells) were separated from 40 or 80 ml. of whole blood by mixing with a fibrinogen-citrate mixture and collecting the leukocyte-rich supernatant. The separated cells were washed three or four times with heparinized Locke-Ringer solution. The final pellet of cells was frozen and

thawed four times and extracted thirty to sixty minutes at 37° C. in Veronal buffer, pH 7.4. The optical density (wave length 550) of the extract was determined. Aliquots of the extracts were then tested for hemolytic and anticomplementary activity in the usual hemolytic system. The results of these tests showed only anticomplementary activity, as evidenced by a decrease in the expected degree of hemolysis in a standard hemolytic test when cell extract was present. In a single experiment, a leukocyte suspension was devoid of hemolytic activity.

DISCUSSION

The destruction of the hemolytic C′ activity of plasma by three separate techniques—heat (C′ 1, 2), zymosan (C′ 3), and ammonia (C′ 4)—did not significantly alter the release of histamine from thrice-washed blood cells resuspended in such plasmas. Similarly, phloridzin did not markedly impair histamine release, although in one experiment in which the concentration was 6.3×10^{-3}M only 73 per cent of control release was obtained. Phloridzin is said to interfere with C′ 3 activity[17] in rat and guinea pig serum. The present results show no striking effect on C′ of human plasma. In the experiments comparing plasma C′ activity with and without histamine release by ragweed antigen, the results did not show any disappearance of hemolytic activity. It would appear, therefore, that C′ is not a necessary component of the release reaction. It could be argued, however, that the minute quantity of C′ remaining among the cells after three washes is all that is required, or that C′ is bound to the cell and cannot be washed away. The experiments do not answer the former argument, but the latter is at least partially answered by the failure to find hemolytic C′ activity in extracts of washed leukocytes (with some erythrocyte contamination). It is possible, however, although it has not been shown here, that C′ might be present in blood cells and that its activity in extracts might be masked by anticomplementary substances.

The failure of iodoacetate inhibition of histamine release to be reflected in any diminution of C′ activity is further evidence against participation of C′ in the release reaction.

Of particular interest is the demonstration that cells washed three times and resuspended in the washing fluid retain the capacity for allergic histamine release. Such systems released 56 to 84 per cent as much histamine as similarly washed cells resuspended in their own plasma. This is a convincing demonstration that allergic sensitizing antibody is firmly bound to the cell and, also, is in keeping with the observation[19] that leukocytes of atopic subjects can be used to sensitize normal skin passively.

SUMMARY

The experiments reported in this article deal with the possible relation of complement (C′) to allergic histamine release from leukocytes of atopic (ragweed-sensitive) persons. The results show that no measurable consumption

of C′ occurs during the release reaction. Systems composed of thrice-washed blood cells reconstituted with plasma which has been treated to destroy hemolytic C′ activity have the capacity to release histamine on introduction of ragweed antigen. Hemolytic activity was abolished by heating at 56° C. for thirty minutes (C′ 1 and C′ 2) and by treatment with zymosan (C′ 3) and ammonia (C′ 4). Washed cells resuspended in such plasmas released 73 to 100 per cent as much histamine as cells resuspended in untreated plasma. Blood cells washed three times in heparinized Locke-Ringer solution and resuspended in this fluid are capable of releasing 56 to 84 per cent as much histamine as washed cells resuspended in their own plasma. Extracts of washed leukocytes (some erythrocytes admixed) obtained after freezing and thawing did not show hemolytic C′ activity but, rather, were slightly anticomplementary, which suggests that C′ is not present in such cell extracts but does not rule out the possibility that anticomplementary effects mask its activity. The inhibition of histamine release brought about by iodoacetate is not accompanied by any change in plasma C′ activity.

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SOME BIOCHEMICAL CHARACTERISTICS OF ALLERGIC HISTAMINE RELEASE FROM LEUKOCYTES OF RAGWEED-SENSITIVE SUBJECTS

Elliott Middleton, Jr., M.D., William B. Sherman, M.D., William Fleming, New York, N. Y., and Paul P. VanArsdel, Jr., M.D., Seattle, Wash.*

IN AN earlier article[1] the results of experiments on the quantitative aspects of histamine release from leukocytes of atopic subjects were presented. It was shown that histamine was released by concentrations of pollen extract as small as 0.01 μg protein nitrogen per liter of blood and that the increase of antigen concentrations produced greater histamine release except in high antigen excess when release was suppressed. The amount of histamine released into plasma was measured by the microchemical method of Lowry and co-workers.[2]

Further experiments have been conducted in an effort to learn more about the nature of the histamine-release reaction. The requirement of divalent cation(s) has been shown, and the effect on the reaction of certain metabolic inhibitors has revealed that iodoacetate and phenol are effective inhibitors. Also, preliminary observations have been made on the effect of temperature on the reaction.

METHODS

The details relating to blood collection, antigen preparation, and histamine determination have been previously described,[1, 2]

Blood Donors.—These were subjects with clinically significant hay fever due to ragweed-pollen sensitivity which was confirmed by intradermal skin test. None had received previous specific hyposensitization with ragweed pollen extract.

Inhibitors.—Table I shows the variety of substances which were tested for their ability to interfere with histamine release. The concentrations studied are given in the table. Oxalate, citrate, and ethylenediamine tetraacetate (EDTA) were dissolved in water and one part was added to nine parts of blood. These concentrations were both anticoagulant and inhibitory. Glycylglycine

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Department of Medicine, University of Washington, School of Medicine.

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buffer, 0.1 M, was used to dissolve the *p*-chloromercuribenzoate and phenylmercuric acetate. Iodosobenzoic acid was dissolved in water by adding 2 N sodium hydroxide in the smallest amount necessary to bring about the solution of the crystals. All the other chemicals listed were dissolved in saline solution. Each reagent, when added, gave the final concentrations noted in the table. The concentrations refer to whole blood without regard to distribution of the compound between cells and plasma or to protein binding.

TABLE I. SUMMARY OF EXPERIMENTS WITH INHIBITORS

INHIBITOR	MOLAR CONCENTRATION OF INHIBITOR*	NO. OF TRIALS	PER CENT INHIBITION†	
			AVERAGE	LIMITS
EDTA	10^{-3}	5	83	74 to 89
Citrate	1.5×10^{-3}	4	86	71 to 93
Oxalate	10^{-3}	3	79	71 to 93
Iodoacetate	10^{-5}	3	0	− 7 to 9
	10^{-4}	3	36	22 to 50
	10^{-3}	6	71	54 to 85
p-Chloromercuribenzoate	10^{-6}	2	2	− 3 to 7
	10^{-5}	5	2	−23 to 32
	10^{-4}	6	6	−26 to 36
	10^{-3}	3	4	−11 to 29
Phenylmercuric acetate	5×10^{-4}	2	10	−10 to 29
	5×10^{-3}	1	31	31
Iodosobenzoate	10^{-4}	1	− 6	− 6
	2×10^{-4}	2	− 5	− 7 to − 3
	10^{-3}	1	−13	−13
Phenol	1.3×10^{-5}	2	− 9	−11 to − 6
	1.3×10^{-4}	2	− 8	−15 to 0
	10^{-3}	6	30	12 to 61
	1.3×10^{-3}	2	43	31 to 65
	10^{-2}	4	46	29 to 90
Salicylate	10^{-3}	4	− 9	−35 to 11
	10^{-2}	2	−20	−25 to −16
Gentisate	10^{-3}	2	10	3 to 16
Benzoate	10^{-3}	2	8	− 3 to 19
	2.5×10^{-3}	1	−17	−17
Phthalate	2.5×10^{-4}	2	17	13 to 21
	10^{-3}	1	22	22
	2.5×10^{-3}	2	23	19 to 27
Phenylbutazone	1.5×10^{-3}	4	26	11 to 39
Fluoride	10^{-4}	3	1	−28 to 23
	10^{-3}	3	3	−10 to 22
	10^{-2}	1	8	8
Cyanide	10^{-3}	3	− 6	−16 to 2
Azide	10^{-5}	3	− 5	−25 to 10
	10^{-4}	4	− 7	−19 to 6
	10^{-3}	5	− 4	−35 to 10
Urethane	10^{-3}	3	1	−21 to 13

*Concentration is expressed as moles per liter of whole blood.

†A minus sign in front of a figure signifies greater than control release of histamine, that is, the reverse of inhibition.

Addition of Inhibitor and Antigen, and Incubation.—Thirty-eight milliliters of heparinized whole blood was collected in siliconized, conical, centrifuge tubes (40 ml.), following which saline control and inhibitor solutions were added and carefully mixed in. Immediately thereafter, freshly diluted ragweed pollen extract was added and all tubes were incubated at 37° C., with occasional mixing, for thirty minutes. In most instances, the final concentration of ragweed pollen was 50 μg protein nitrogen per liter of whole blood. (In several cases it was less because lower concentrations gave maximum release.) Following the incubation period, the tubes were centrifuged, and plasma aliquots were obtained for histamine analysis. The percentage of inhibition was calculated by subtracting from 100 the per cent of control release in the presence of inhibitor. Because of the inherent variabilities of this complex biologic system and the range of error of the analytical method, we have rather arbitrarily considered inhibition to be real and significant when reproducibly greater than 25 to 30 per cent. Histamine-recovery experiments were done in the presence of each inhibitor to ensure against false positive results due to interference with the analytical method.

The effect of temperature on the release reaction was studied by first bringing pairs of tubes containing the blood to temperatures between 14° and 45° C. and then adding saline solution and antigen. The tubes were kept at these temperatures for thirty minutes, after which they were promptly centrifuged and the plasma was collected and analyzed.

RESULTS

From the data presented in Table I, it is clear that the chelating agents, EDTA, oxalate, and citrate, are very effective inhibitors of histamine release. Inhibition occurred to the extent of 79 to 86 per cent. Recalcification of EDTA-anticoagulated systems returned the capacity to release histamine. This was demonstrated in two experiments by adding 1 ml. of 1 M calcium chloride to EDTA-anticoagulated blood containing a small amount of heparin, following which addition of antigen caused release of 53 and 61 μg of histamine per liter of plasma. The controls to which no calcium had been added did not show histamine release. (This finding prompted further experiments on the role of calcium and of magnesium ions in the release reaction which will be presented in a subsequent report.)

Iodoacetate was another consistently effective inhibitor. The degree of inhibition increased with increasing concentration. At a concentration in whole blood of 10^{-3} M, the average inhibition was 71 per cent.

In other experiments, as tabulated, phenol was found to inhibit release. At a concentration of 1.3×10^{-3} M, this occurred to the extent of 31 to 65 per cent. This finding is of practical as well as theoretical interest, since extracts containing 0.5 per cent phenol, if added to 40 ml. whole blood, will give a final concentration of 1.3×10^{-3} M. Concentrations of phenol which are less by at least one order of magnitude are not inhibitory and, conversely, the greatest

degree of inhibition was found with 10^{-2} M phenol. Even this high concentration produced an average inhibition of only 46 per cent, considerably less than that caused by iodoacetate and the chelating agents.

Two other compounds, phenylbutazone and phthalate, showed consistent inhibitory properties, although of mild degree. In four experiments with phenylbutazone at a concentration of 1.5 x 10^{-3} M, the average inhibition was 26 per cent. Average inhibition with phthalate (fewer experiments) was 17 to 23 per cent at concentrations ranging from 2.5 x 10^{-4} M to 2.5 x 10^{-3} M. These levels of inhibition are of borderline significance.

Of all the other compounds studied, none showed a reproducible capacity to block histamine release. On the contrary, it appeared in some experiments (for example, those with fluoride, azide, and salicylate) that there was some potentiation of histamine release by ragweed antigen.

The effect of temperature on the reaction is shown in Table II. It can be seen that there is a marked depression of release in all experiments in which cells were kept at 45° C. during the incubation period. In four of the experiments, there was a diminution of release at temperatures in the 14° to 26° C. range as compared with the release at 37° C. Future experiments on the velocity of histamine release at different temperatures will permit a calculation of the temperature coefficient of the reaction.

TABLE II. EFFECT OF TEMPERATURE ON HISTAMINE RELEASE

SUBJECT	CONCENTRATION OF RAGWEED*	HISTAMINE RELEASED (μG/L. PLASMA) AT VARIOUS TEMPERATURES (DEGREES C.)							
		14°	16°	20°	26°	30°	37°	40°	45°
Bel	50	9		17			63		0
Bor	50						41	52	1
Mor	50				5	16	16	20	2
Gor	50		0		0		14		0
Deu	50				81	84	81	74	6
Pal	50	26					33		
	5	7					26		

*Micrograms of ragweed protein nitrogen per liter whole blood.

DISCUSSION

The results of these experiments indicate that allergic histamine release from leukocytes of atopic subjects (ragweed allergy) following interaction of antigen, antibody, and cells can be blocked by the chelating agents, EDTA, oxalate, and citrate, and also by iodoacetate and phenol. The exact biochemical events affected by these compounds remains to be elucidated, however. Histamine release is also abolished by heating cells to 45° C.

The concentrations of EDTA, oxalate, and citrate which were anticoagulant were found to be inhibitory. This finding strongly suggests the requirement of some divalent cation(s), most likely calcium. Calcium ions are necessary for anaphylactic histamine release from platelets of rabbit blood and whole blood, as reported by Humphrey and Jacques[3] and by McIntire, Roth, and Richards.[4]

That histamine release occurs following recalcification of EDTA-anticoagulated whole blood indicates the reversibility of EDTA inhibition. Both Humphrey and Jacques[3] and McIntire and associates[4] found that citrate produced an irreversible inhibition, however, suggesting a mechanism of inhibition other than cation exclusion. (Although we have not investigated this aspect in detail, it is worth mentioning that in two of three recalcified citrated systems in which heparin was present for anticoagulation histamine release after addition of antigen was moderately reduced.)

The well-known role of calcium and magnesium in complement fixation and immune hemolysis causes speculation as to the role of complement (C′) in allergic histamine release in the human being. Indeed, the requirement of some heat-labile plasma factor other than antibody was shown in the experiments of Humphrey and Jacques.[3] Other animal experiments, notably those involving passive cutaneous anaphylaxis[5, 6] and anaphylatoxin generation,[7] have implicated the participation of C′ in hypersensitivity reactions. In experiments reported in the accompanying article, however, we have evidence that histamine release is not mediated by C′ and hence that the calcium dependency is not related to C′ activity. This is in keeping with the findings of Mongar and Schild[8] with respect to anaphylactic histamine release from minced, sensitized lung of the guinea pig.

Inhibition of histamine release by iodoacetate was a consistent and reproducible phenomenon, and this correlates well with the findings of Mongar and Schild.[9] Iodoacetate is well known as a sulfhydryl enzyme inhibitor,[10] but it is also known to react with chemical groupings other than sulfhydryl.[11] It is possible, therefore, that it may inhibit histamine release by some other mechanism.

The other sulfhydryl reagents,[10] *p*-chloromecuribenzoate, phenylmercuric acetate, and iodosobenzoate, failed to produce consistent inhibitory effects. This is not in agreement with findings reported by the British workers and throws doubt on, but does not rule out, the possibility that the release of histamine in allergic human beings is dependent on some sulfhdyryl enzyme system.

Phenol was another compound with definite inhibitory effects on histamine release. Phenol has antipyretic activity but is not used because of its toxicity. Although no definite information is available regarding the histamine release system of the human being, evidence from animal experiments[9] suggests that phenol does not interfere with the antigen-antibody reaction nor does it inactivate antibody or prevent desensitization. It thus appears to uncouple or disassociate the antigen-antibody reaction from the histamine-release reaction. Testing such effects in the human blood system is not feasible, since washing out the phenol and restoring the cells to the pretreatment state are technically difficult.

In keeping with the findings of Mongar and Schild is the inhibition of release obtained with phenylbutazone. This compound was an active inhibitor

in their tests on the minced guinea pig lung system at concentrations of 1 to 2 x 10^{-3} M. In the human allergic histamine-release system, however, a concentration of 1.5 x 10^{-3} M produced an average inhibition of 26 per cent.

The other compounds studied, including antipyretics (salicylate and benzoate), antirheumatics (*p*-aminobenzoate, gentisate, and phthalate), respiratory enzyme inhibitors (cyanide, fluoride, and azide), an anesthetic agent (urethane–studied because of its known depressant action on smooth muscle contraction in anaphylaxis), all failed to produce significant and reproducible inhibition. Mongar and Schild[8] found many of these substances to be inhibitory, but most often at higher concentrations than those used in our experiments. They also noted that concentrations which were inhibitory also depressed oxygen consumption.

The results obtained in our heating experiments are entirely in accord with the findings of Mongar and Schild, that is, there is virtually complete absence of histamine release when blood is heated to 45° C. In our experiments, however, the release reaction was carried out at 45° C., blood not being cooled to 37° C. before addition of antigen. The reversibility of the influence of high temperature has not been studied. If, however, the parallel with anaphylactic release from guinea pig lung is quite complete, then it may be assumed that cells heated to 45° C. undergo an irreversible change, presumably enzyme inactivation, which precludes allergic histamine release.

SUMMARY

The experiments reported in this article show that allergic histamine release from leukocytes of atopic subjects (ragweed sensitivity) can be blocked by the chelating agents, EDTA, citrate, and oxalate. The concentrations which are anticoagulant also inhibit histamine release. This finding is interpreted as showing a requirement for calcium ions in the release reaction.

Of a number of other compounds tested for their inhibitory effect on histamine release, only iodoacetate and phenol were found to be consistently inhibitory. Phenylbutazone and phthlate exhibited mild inhibitory properties. The mechanism by which these compounds inhibit has not been elucidated.

Allergic histamine release is abolished when the reaction is run at 45° C., and it is reduced at temperatures below 37° C.

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FATAL NECROTIZING ANGIITIS ASSOCIATED WITH HYPERSENSITIVITY TO PENCILLIN O AND IODIDES

Report of Case

Gustavus A. Peters, M.D., Roland W. Moskowitz, M.D., Louis E. Prickman, M.D., and Haddon M. Carryer, M.D., Rochester, Minn.*

UP TO A FEW YEARS AGO, periarteritis nodosa was rare and its cause unknown, but with more cases being reported and studied there appears to be little doubt that the condition is sometimes due to hypersensitivity reactions. This concept was suggested by Gruber[1] in 1925 and later substantiated by the work of Rich,[2] Rich and Gregory,[3] Clark and Kaplan,[4] and Germuth.[5] According to Collens and Wilensky,[6] most of the evidence at present suggests that periarteritis nodosa is fundamentally an antigen-antibody reaction involving the blood vessels. However, the groundwork for considering periarteritis nodosa more definitely on the basis of hypersensitivity created by drugs goes back to the fundamental studies of Landsteiner and Chase[7] with haptenes in the production of anaphylactic sensitivity.

Because of the varied morphology of periarteritis nodosa, Zeek[8] has preferred to use the term "necrotizing angiitis" to cover the entire group of vascular lesions, both arterial and venous, in which the fully developed stage is characterized by fibrinoid necrosis and inflammation. She distinguishes five different types morphologically and clinically, namely (1) hypersensitivity angiitis, (2) allergic granulomatous angiitis, (3) rheumatic arteritis, (4) periarteritis nodosa, and (5) temporal arteritis. The first two, she has said, may be due to hypersensitivity, but in classic periarteritis nodosa, hypertension is considered to be the important factor. While her classification is attractive, there is difficulty at times in recognizing each type pathologically and clinically.

Hypersensitivity appeared to play a definite role in the case of necrotizing angiitis that we are reporting. The case is unusual in that (1) it is one of the very few in which penicillin O has been suspected as the cause, (2) it was further complicated by the development of iodide sensitivity, (3) it may represent cross sensitization to penicillin O or a combination of sensitivity to both penicillin and iodides, and (4) it was not characterized by any healing in the arterial walls following cortisone therapy, which may well indicate that the dosage used was inadequate.

From the Section of Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minn.
The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.
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*Fellow in Medicine.

REPORT OF A CASE

A 39-year-old white housewife, mother of seven children, who came to the Mayo Clinic on Nov. 8, 1953, had bronchial asthma, vasomotor rhinitis, nasal polyps, and bilateral chronic maxillary sinusitis. She had a history of being allergic to penicillin since 1950. The ear, nose and throat consultant recommended that she have bilateral nasoantral windows made, as well as nasal polypectomy and ethmosphenoidectomy. These procedures were carried out on November 13 after cortisone preparation for 2 days prior to operation. Preparation with cortisone was indicated because she had had 20-tablet courses of cortisone therapy 12 months, 5 months, and 3 months previously for relief of asthma. The amount of cortisone acetate used was 200 mg. intramuscularly 2 days before, 200 mg. the day before, and 100 mg. the day of operation. Postoperatively, the amount was rapidly reduced to none, and radium was applied to the nasal cavities in an attempt to prevent recurrence of polyps.

Fig. 1.—Appearance of bullae on face.

Because of her known sensitivity to penicillin G, she was given penicillin O, on the theory then current that it was hypoallergenic, in doses of 1,000,000 units along with 0.5 Gm. of streptomycin intramuscularly daily for 6 days postoperatively. In addition, she was given a nasal aerosol containing penicillin O and variable quantities of streptomycin, amounting to a total of 700,000 units of penicillin O and 0.0675 Gm. of streptomycin. The aerosol was mixed with Alevaire and administered at 2-hour intervals day and night for 6 days. There was no apparent immediate untoward reaction and she was dismissed a week later.

In December, 1953, she had pneumonia and was treated by her family physician with "nonallergenic" penicillin (O), approximately three or four injections, as well as with chlortetracycline (Aureomycin), four capsules daily for 1 week. At that time she said that her nose had continued to feel "plugged and stuffed" since her nasal operation. She recovered from this infection but was admitted to our dermatology service on Jan. 8, 1954, because of an eruption of the hands, face, and elbows of 3 weeks' duration. She had first noticed an erythematous papule over the dorsum and knuckles of the right hand.

Later bullae appeared, and, after a few days, vesicles were evident on the opposite hand as well as on the arms and legs. Bullae appeared on her face after her admission to the hospital (Fig. 1). She remained in the hospital for 6 days and was dismissed with a diagnosis of erythema multiforme. Her skin cleared and remained so until January 19, when a few hours after taking a teaspoonful of an iodide mixture for her asthma she broke out again with a skin reaction similar to the one she had presented on January 8 (Fig. 2). She was advised to avoid iodides in the future.

The patient remained fairly well, though weak and "run down," until about February 1, when she contracted a cold following chilling in subzero weather. Her asthma recurred and necessitated hospitalization in her home community where she again received penicillin O, cortisone, epinephrine, oxygen and other supportive agents. About February 20, she began to have soreness in the calves of her legs when walking, edema of the ankles and hands, burning in her feet and hands, and weakness of the hands and legs. Arthralgia

Fig. 2.—Appearance of eruption on hands.

was present in the wrists and ankles. About March 4, purpuric and ecchymotic areas developed about the ankles, feet, hands, sacrum, buttocks, and right ear. She was hospitalized in her home community on March 6 and was treated with vitamins K and B_{12}. Her asthma recurred and she was transferred to the Rochester Methodist Hospital 9 days later, where she was under observation and treatment until her death on April 15, 1954.

When the patient was admitted on March 15, 1954, the condition of her legs seemed to be somewhat improved. The burning had decreased considerably, though pruritus remained, and the edema was subsiding; however, she was still weak and clumsy. Her hands were swollen, stiff, weak, and incoordinated. Her blood pressure was 120 mm. Hg systolic and 80 mm. Hg diastolic, her temperature 98.2° F., and her pulse rate 140 per minute. She appeared thin, pale, and chronically ill. She had a purpuric and ecchymotic eruption about the right ear, hands, sacrum, buttocks, ankles, and feet. Some old vesicular lesions on the legs appeared to contain pus. She had a creamy yellow discharge from the left external auditory meatus and a purulent postnasal discharge. Sibilant and sonorous râles were heard over the lung fields on expiration and inspiration. Expiration was prolonged, and chest expansion was limited. The right hand, forearm, and elbow were swollen. The fingers were stiff. Weakness of the arms and legs was believed to be the cause of her incoordination. The anteroinferior portion of the left tympanic membrane was perforated; the final diagnosis was chronic suppurative otitis media.

Dermatologic examination revealed that the skin reaction was somewhat different from the iodide eruption seen in January. The latter had cleared in 6 days, while the present one had been evident for 10 or 12 days and was hemorrhagic. It was thought that penicillin was most likely responsible unless the patient had taken iodides while in the hospital in February. The urticaria had been present about 3 days. The consultant in hematology believed that the patient had a vascular type of purpura, probably part of a sensitivity reaction, and noted that the high eosinophilia (55 per cent) was unusual although seen in some asthmatics in whom periarteritis nodosa develops. We who saw the patient clinically thought she had a drug reaction to penicillin O, although evidently she had no apparent immediate reaction to it when it was given in November, 1953. However, the preoperative administration of cortisone may have protected her. Penicillin G had provoked asthma in the preceding 3 years. Results of the lupus erythematosus clot test were negative.

On March 18, 1954, a diagnosis of periarteritis nodosa secondary to penicillin O was made and cortisone therapy was begun, starting with 200 mg. a day and later decreasing to 75 mg. a day. On March 23, a neurologic diagnosis of polyneuritis of the allergic type was made. Serum was obtained before cortisone therapy was started, and passive transfer studies with this in normal donor's skin showed no reactivity to penicillin G, penicillin O, or procaine. No tests were done with iodides as an antigen. Following the use of cortisone the patient seemed to gain in strength for 2 or 3 days, when supraventricular tachycardia (rate of 164) with palpitation and weakness developed unexpectedly. Quinidine and digitalis were given. About this time the urine began to show albumin and blood cells. Also, the blood pressure rose to hypertensive levels. The retinal arterioles showed narrowing, grade 2, with focal constrictions, grade 1, but no sclerosis. Vision was good.

Pain in the extremities continued to grow worse, requiring treatment with meperidine (Demerol) and morphine. On April 6, biopsy of the left gastrocnemius muscle confirmed the clinical diagnosis of periarteritis, and a bone-marrow study 4 days later showed severe eosinophilia and perivascular cuffing with plasma cells. On April 8 the patient had some epigastric and right upper quadrant distress with resistance on palpation in these regions. She also had dysphagia and was thought to be having intracranial involvement. The pain in the legs became almost intolerable, and any activity made it worse. Two or 3 days previously, her voice had become hoarse and the question of vocal cord weakness arose. Her urine showed a progressive increase in number of erythrocytes and in albumin. More petechiae developed and the abdomen became distended.

On April 14 the patient became disoriented and had visual hallucinations. Her blood pressure increased to 208/120 mm. Hg, her pulse rate increased to 124 per minute, and later some jerking developed in her left leg and arm. She became comatose and died on April 15, the terminal episode being due to cerebral involvement.

X-ray and laboratory data are given in Table I. In November, 1953, and January, 1954, routine urinalyses showed no blood cells. On March 16 the urine showed microhematuria, grade 1, and this increased up to the time of her death. Albuminuria appeared on March 31 and persisted until death. The value for hemoglobin decreased somewhat from November, 1953, to April, 1954. Leukocytosis was present from January, 1954, reaching its maximum about the middle of March. The differential counts showed marked eosinophilia most of the time, reaching its peak about the time the patient's condition was getting worse. The sedimentation rate was high even in January when she had the skin eruption probably due to iodides, then dropping until the terminal phase. The values for electrolytes were essentially normal up to within 1 week of death. Her temperature during her last hospitalization, as well as during the other two, remained normal or subnormal until the final day of life. Her coagulation and prothrombin times were within normal limits but the bleeding time was slightly increased. Inoculation of rabbit cornea with fluid removed from one of the bullae when she had a reaction to iodides in January did not produce any evidence of herpes virus. The results of all routine skin tests for allergy were negative except for a grade 3 reaction to cattle hair.

TABLE I. X-RAY AND LABORATORY DATA

ITEM	YEAR 1953	YEAR 1954						
	11-10	1-9	3-16	3-20	3-27	3-31	4-3	4-10
Thoracic roentgenogram	Neg.	Neg.	Neg.					
Urine								
Pyuria (grade)	*	1	1			4	2	1
Erythrocyturia (grade)			1			2	2	2
Albuminuria (grade)						2	2†	3
Blood								
Hemoglobin (Gm./100 c.c.)	13.2	12.8	11.9‡	11.2		12.2		11.2‡
Leukocytes (1,000s/c. mm.)	10.4	12.3	81.0	58.0	27.1	25.6		17.0
Differential count (%)								
Lymphocytes	26.5	10.5	10	9	8	13		16
Monocytes	4.5	4.0	4	2	5	4		2
Neutrophils	42.5	30.5	31	29	24	39		54
Eosinophils	25§	55	55	55§‖	61§‖	44		28
Sedimentation rate (mm. in 1 hr.)¶		75#		47	30	27		47
Flocculation test for syphilis	Neg.			Neg.				

*Occasional pus cell.

†Also grade 1 cylindruria (granular).

‡Erythrocyte count, 4,270,000 and 3,820,000 on 3-16-54 and 4-10-54, respectively.

§Also basophils, 1.5, 2, and 1 per cent on 11-10-53, 3-20-54, and 3-27-54.

‖Also myelocytes, 3 and 1 per cent on 3-20-54 and 3-27-54.

¶Other blood data on 3-16-54: platelet count 294,000, bleeding time 7.5 min., coagulation time 6.25 min., clot retraction time 2 hr., and blood smear showing extreme eosinophilia. On 3-19-54: result of LE clot test negative. On 4-2-59: blood urea 28 mg./100 c.c., serum sodium 131, potassium 4.5, and chlorides 90.8 mEq./L.

#Sedimentation rate was 71 and 38 mm. on 1-12-54 and 3-19-54.

It seemed, in retrospect, that most of the vascular and renal changes had come in the final 4 weeks. Her progress had appeared to be satisfactory until she had become overly tired one day, and following this she started the downhill trend, but this may have been coincidental.

At necropsy, external examination revealed ecchymotic areas on the thighs, arms and shoulders, and petechiae over the abdomen, thighs, legs and feet. In the heart and pericardium were many small nodules 1 to 2 mm. in diameter, firm and yellow, and situated along the small vessels of the pericardium. Small focal infarcts were present throughout the myocardium. Hemorrhagic areas, a few millimeters in diameter, were seen throughout both upper lobes of the lungs, while small infarcts could be seen and palpated over the surface of both lower lobes. The bronchi were prominent, and bloody mucus and pus were present in both the trachea and the bronchi. Bronchiectasis was noted in the right upper lobe and both lower lobes, being most marked in the right lower lobe. Small hemorrhages could be seen throughout the spleen, and nodules were palpable along the splenic vessels. No evidence of infarcts was grossly visible. Perivascular nodules and small areas of infarction were evident in the liver. The gall bladder showed on its surface many nodules 1 to 3 mm. in diameter, along the course of the small vessels.

A mass approximately 5 cm. in diameter and hemorrhagic in appearance was present along the lesser curvature of the stomach; it proved to be a hematoma containing clotted blood. An ulcer, measuring 1 by 2 cm., was seen on the posterior wall of the stomach, approximately 5 cm. proximal to the pylorus. The small intestine and the colon contained many small infarcts, varying in diameter from a few millimeters up to 2 cm.; these were most abundant in the lower part of the ileum. The pancreas manifested nodularity similar to that seen in the heart, spleen and liver. There was bilateral cortical atrophy of the adrenals, and the cortices appeared rather pale. The kidneys were pale and had numerous petechiae over their surfaces. No evidence of gross infarction was seen. Palpation revealed small nodules in the bladder and diaphragm. The breasts, thyroid, and parathyroids appeared grossly normal.

A hemorrhagic infarct involved the right frontal lobe of the brain, and another involved the right temporal lobe. The fourth ventricle was filled with blood, and subarachnoid hemorrhage was seen over the spinal cord.

Histologically, numerous lesions of necrotizing arteritis were found throughout many of the organs studied, including the kidneys, heart, gastrointestinal tract, gall bladder, pulmonary-artery branches, follicular arterioles of the spleen, liver, pancreas, muscle, adrenal glands, uterus, bladder, peripheral nerves, and central nervous system. Arterioles and capillaries were commonly involved as well as small and medium-sized arteries. Two types of arteritis were seen. In the lungs, peripheral nerves, liver, and gastrointestinal

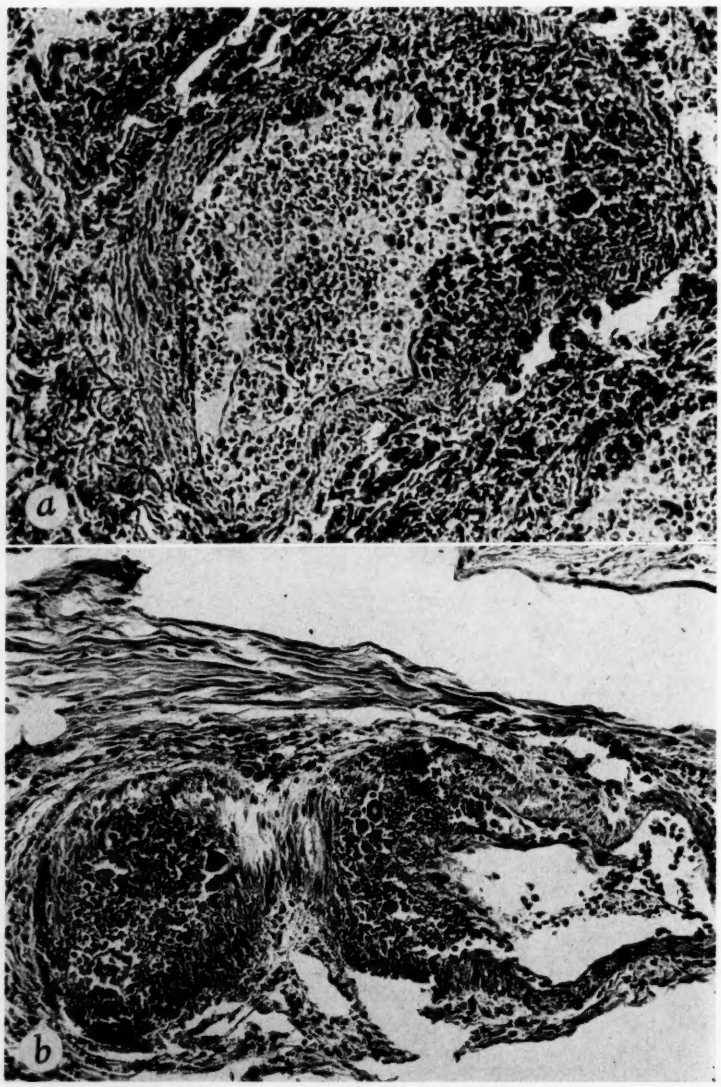

Fig. 3.—*a*, Small pulmonary artery showing granulomatous arteritis. Extensive inflammatory exudate consisting mainly of round cells and an occasional giant cell replaces much of intima and media in one part of vessel wall (hematoxylin and eosin; × 200). *b*, Artery from right sciatic nerve showing granulomatous arteritis. Inflammatory exudate has destroyed much of intima and media at vessel branching. Giant cell reaction is prominent (hematoxylin and eosin; × 150).

tract were seen vessels with granulomatous inflammation. The inflammatory reaction was predominantly intimal or adventitial and consisted of varying numbers of lymphocytes, plasma cells, polymorphonuclear leukocytes, epithelioid cells, and giant cells of foreign body or Langhans' type (Fig. 3). Eosinophilic infiltration was prominent in many areas of arterial inflammation. Other vessels in the organs noted showed the more characteristic acute arteritis as seen in typical cases of periarteritis nodosa. This consisted of fibrinoid necrosis of the vessel wall with acute inflammatory exudate throughout the various coats of the wall (Fig. 4, *a*). All the lesions seen were of either the acute or the subacute type corresponding in characteristics to the stages of acute inflammation or stage of granulation inflammation as described by Arkin.[9] No extravascular granulomas were seen, but extravascular inflammatory reactions of other forms were notable.

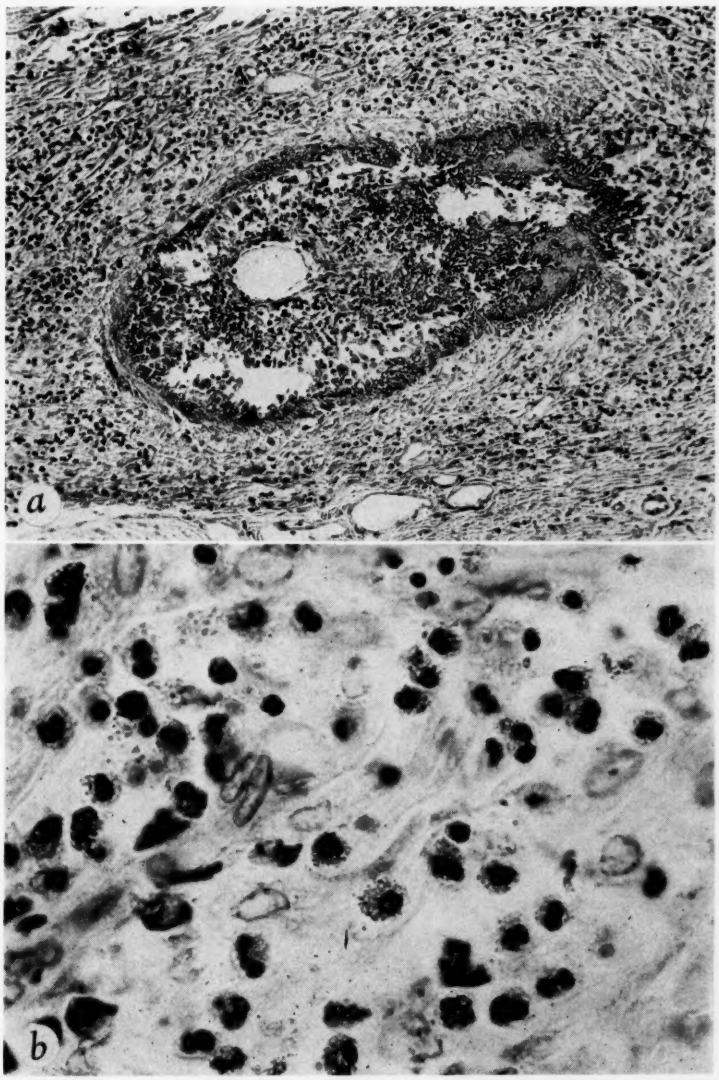

Fig. 4.—*a*, Artery in liver showing acute arteritis. Fibrinoid necrosis has destroyed much of media. Intima manifests inflammation with polymorphonuclear and round cells (hematoxylin and eosin; × 95). *b*, Interstitial inflammatory reaction in hilus of liver. Inflammatory response is almost entirely eosinophilic (hematoxylin and eosin; × 725).

Interstitial inflammation was seen in areas of the heart, liver, and peripheral nerves (Fig. 4, *b*). The inflammatory exudate was predominantly eosinophilic and plasma cell in character. In the cardia of the stomach there was an interstitial inflammatory nodule consisting of a central core of pyknotic nuclei and some fibrinoid material surrounded by macrophages which were in turn surrounded by an inflammatory exudate of eosinophils, lymphocytes, and plasma cells. In the kidneys were somewhat similar focal areas having a fibrinoid center with mononuclear inflammatory reaction surrounding it (Fig. 5).

Numerous areas of fibrinoid necrosis of glomerular tufts of the kidneys were seen (Fig. 6, *a*). Epithelial proliferation of the glomeruli was prominent, with adhesive capsulitis and crescent formation (Fig. 6, *b*). Periglomerular inflammatory reaction with polymorphonuclear leukocytes and lymphocytes was common. Occasional glomeruli showed areas of fibrosis. Tubules manifested severe degenerative changes. In the interstitial tissues a prominent inflammatory reaction with lymphocytes and plasma cells was seen.

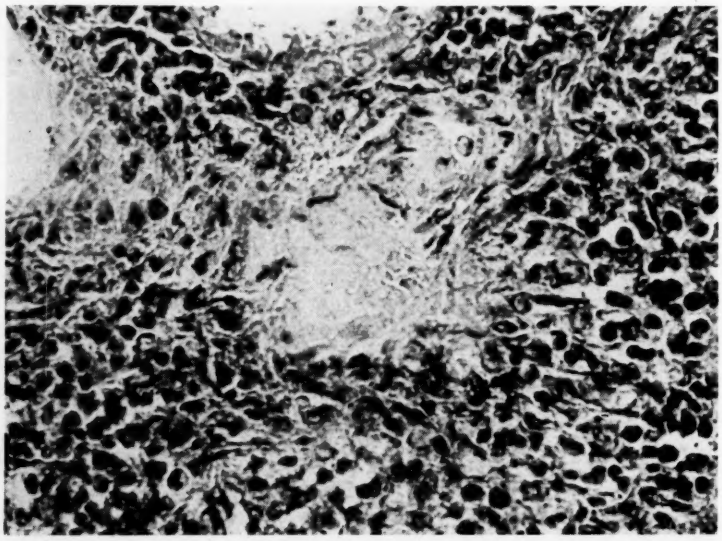

Fig. 5.—Extravascular inflammatory reaction in interstitial tissue of kidney. Central core of fibrinoid necrosis is surrounded by a broad zone of macrophages, round cells, and polymorphonuclear cells (hematoxylin and eosin; × 300).

Associated pathologic findings included diffuse areas of bronchopneumonia, bronchiectasis, and changes consistent with chronic asthmatic bronchitis. The appearance of the gastric ulcer noted on gross examination was not inconsistent with that seen in gastric ulcerations complicating steroid administration. A number of small arteries and arterioles in the submucosa and adventitia in the area of the gastric ulcer were involved with acute arteritis and hyalinized thrombi. In the area of hematoma in the stomach an artery showed extensive necrosis. In one area of this vessel there was a deficiency in a segment of the wall through which the lumen communicated with the surrounding hematoma (Fig. 7). The nasal polyp was not unusual and contained no arterial lesions. Numerous lesions of acute arteritis were evident in the central nervous system, predominantly in the pia-arachnoid, although occasional involvement of vessels was seen within the brain substance itself. The adrenal cortices showed atrophy consistent with long-term administration of steroids.

The anatomic diagnoses were: (1) periarteritis nodosa (drug allergy); (2) intracerebral hemorrhage, right frontal, and infarct, right temporal lobe; (3) subarachnoid hemorrhage with hemorrhage in the fourth ventricle; (4) infarcts of the intestines, lungs, and brain; (5) hypertrophic bronchitis (asthma clinically); (6) purulent bronchitis with bronchiectasis; (7) pulmonary emphysema; (8) atrophy of the adrenal glands (cortisone

therapy); (9) hemorrhages in the skin (questionable nodular lesions); (10) periarteritis of the gall bladder; (11) hypertrophy of the kidneys; (12) grade 1 arteriosclerosis of the aorta, coronary arteries, and cerebral vessels; (13) ulcer of the stomach; (14) hematoma in the lesser curvature of the stomach; and (15) ulcer of the gall bladder.

Comment on Pathologic Findings.—Numerous reviews of the pathology of periarteritis nodosa are presently available in the literature.[9-13] Studies within the past 2 decades have stressed the hypothesis that many cases formerly included under the broad classification of periarteritis nodosa are actually cases of allergic or hypersensitivity phenomena.[14-16] Some observers think that the pathologic picture of periarteritis nodosa differs from that of hypersensitivity

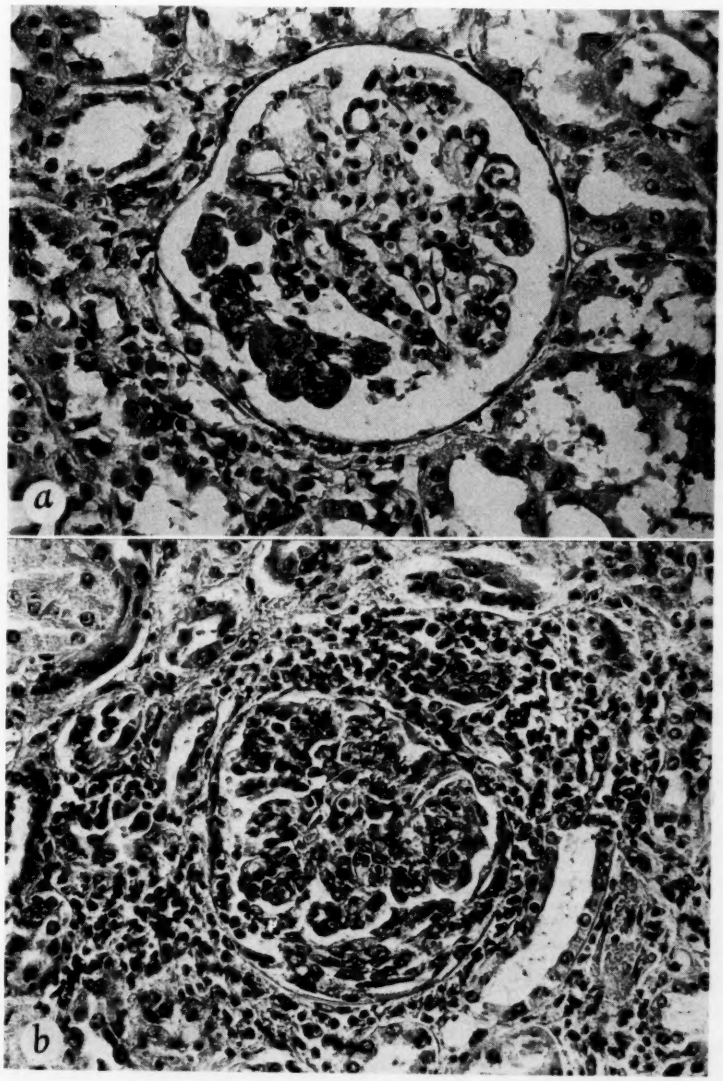

Fig. 6.—Glomeruli of kidney. *a*, Fibrinoid necrosis involving focal areas of glomerular tuft (hematoxylin and eosin; ×300). *b*, Prominent epithelial proliferation with crescent formation, and periglomerular inflammatory reaction with lymphocytes and polymophonuclear cells (hematoxylin and eosin; × 300).

angiitis.[17] In periarteritis nodosa, primarily the small and medium-sized muscular arteries are affected. The lesions are of varying ages from acute to healing, involvement of pulmonary-artery branches and splenic follicular arterioles is infrequent, and granuloma formation with epithelial cells and giant cells is extremely rare. Allergic angiitis, on the other hand, is characterized by involvement of small arteries, venules, and arterioles. Involvement of pulmonary-artery branches and splenic follicular arterioles is common, all the lesions are of a similar age (usually acutely exudative in appearance), and necrotizing glomerulonephritis is frequently found. Case reports of angiitis following drug reactions stress "extravascular" lesions, including focal necrosis, focal granulomas, interstitial inflammatory lesions

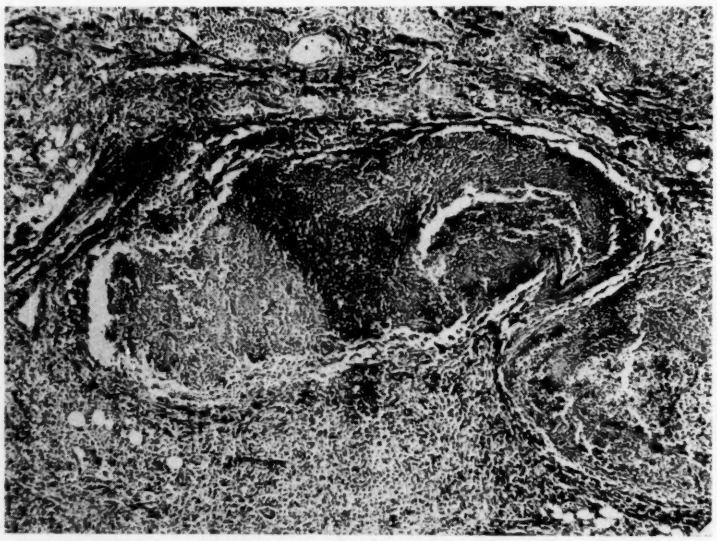

Fig. 7.—Artery in stomach showing extensive necrosis of arterial wall. Communication of vessel lumen with surrounding hematoma due to deficiency in a segment of the wall is seen (hematoxylin and eosin; × 57).

(especially of the heart and kidney), and prominent eosinophilia.[18-20] Churg and Strauss[20] delineated a further division of necrotizing angiitis, termed "allergic granulomatous angiitis." This entity is characterized pathologically by extravascular inflammatory lesions, focal and diffuse, as well as extramuscular granulomas. The vessels show the usual vasculitis seen both in periarteritis nodosa and in granulomatous arteritis. In the cases of Churg and Strauss, all stages of lesions were seen, but healing lesions predominated. Veins were not uncommonly involved.

In the present case there were lesions that fit fairly well into the category of both allergic angiitis and allergic granulomatous angiitis. This is not unexpected in that allergic granulomatous angiitis is a combination of the lesions of allergic vasculitis and allergic granulomatosis. The clinical history of multiple drug reactions, as well as certain facets of the histologic picture, including acute rather than healed lesions and necrotizing glomerulitis, favors

this being truly an allergic angiitis. On the other hand, the history of long-term clinical asthma and marked eosinophilia, along with histologic evidence of granulomatous polyarteritis and extravascular inflammatory lesions with fibrinoid center, supports the view that this was a case of allergic granulomatous angiitis. That these views are not inconsistent has been noted above, and actually supports the concept that various forms of necrotizing angiitis are merely stages in a spectrum of responses to similar pathogenic stimuli. In either case, the over-all pathologic picture supports the idea that an allergenic agent was responsible for the patient's clinical picture.

Incidentally, the renal findings are consistent with the picture of focal necrotizing glomerulitis as described by Davson and associates[21] and Wainwright and Davson.[22] In their cases presenting this lesion, the remainder of the findings were those primarily of allergic angiitis, not unlike those recorded in the present case.

COMMENT

This case of necrotizing angiitis is similar to those reported by Rich[2] and occurring after the use of sulfonamides and heterologous serum, with the finding of collagenous degeneration and arterial lesions showing hyaline and fibrinoid changes microscopically. We believe that penicillin O was probably responsible for the development of the necrotizing angiitis and that for this patient this drug was not hypoallergenic. However, the sensitivity to iodides cannot be entirely discounted. Perhaps the groundwork for hypersensitivity reaction was being laid by the administration of penicillin O, and sensitivity to iodides may have been the basis for the incident that started the patient to react systemically, culminating in the necrotizing angiitis found generally. If this case is one of hypersensitivity to penicillin O, it illustrates that cross sensitization can and does occur with related drugs. The case illustrates that it is best to avoid similar drugs in a patient who has been sensitive to a drug, and a drug with a different chemical structure should be used.

Another point that needs emphasis in this case is that the dosage of cortisone probably was not large enough to ensure healing of the arterial lesions. Some investigators advocate very large doses, as much as 0.5 to 1 Gm. daily, in order to produce healing of such lesions. In retrospect, it would appear that this patient was not doing well in January, 1954, since at that time her blood was showing evidence of abnormality in that she had leukocytosis, an increased sedimentation rate, and a high eosinophilia (55 per cent). The groundwork for the systemic condition may have been present at that time. Her resistance appeared to be low, as shown by the fact that she was having recurrent upper respiratory infections. With cortisone therapy, such infection could have been masked further. At any rate, drug reactions can be serious, and may be fatal, as is illustrated by this case. It is our conviction that internists should always be on the alert for drug sensitivity and be prepared to treat it, as well as to avoid continuation of the use of drugs that may produce hypersensitivity reactions similar to those in this case.

SUMMARY

A case of fatal necrotizing angiitis is thought to have resulted from the administration of penicillin O to a patient known to be sensitive to penicillin G. The patient also had an iodide allergy and hence it cannot be stated definitely that this did not have a place in the production of the necrotizing angiitis. Several of us who saw the patient thought that the penicillin O was probably more important than the iodides in the final outcome of the case. Postmortem findings were typical of allergic angiitis described by Zeek, while some features were characteristic of the allergic granulomatous type described by Churg and Strauss. Skin reactions to penicillin G and O and procaine were all negative. The patient died despite cortisone therapy; the dosage level may have been inadequate. This case, as well as others which have been described in the literature, especially those of Gruber and Rich, shows that drug allergy is important in the pathogenesis of some of the so-called cases of periarteritis nodosa.

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DELAYED HYPERSENSITIVE REACTIONS: A GROWING CLASS OF IMMUNOLOGIC PHENOMENA

Byron H. Waksman, M.D., Boston, Mass.*

IT IS conventional to regard most or all immunologic reactions as being mediated by circulating (humoral) antibody. However, it has long been recognized that other unidentified mechanisms must play a role in the genesis of the reactions of bacterial allergy (of which tuberculin sensitivity is the prototype) and in contact allergy, since no antibody could be identified as responsible for sensitivity in these cases and since passive transfer with serum, even from highly sensitive donors, was ineffective. These reactions have usually been given the generic name "delayed" since their time course is measured in days instead of in minutes or hours, as are the anaphylactic and Arthus responses. In recent years, new evidence has permitted a more exact characterization of reactions of the delayed type as being cellular responses analogous to the cellular response accompanying antibody formation and not events secondary to an antigen-antibody combination in the tissues. It has also become clear that other important categories of immunologic response must be included in this group, among them the rejection of solid-tissue homografts, the experimental "autoallergies," certain experimentally produced inflammatory lesions of connective tissue, and delayed responses to pure proteins.

The experimental properties which characterize the state of delayed hypersensitivity are as follows:

1. Delayed sensitization is difficult to induce by conventional methods for producing circulating antibody. Infection with living cells, the use of mycobacterial adjuvant in mineral oil, the use of very small doses of antigen, and, in particular, the use of the intradermal route of inoculation have been established as especially effective techniques. The use of the intravenous, intraperitoneal, intramuscular, or subcutaneous routes and of large doses of antigen without adjuvant, while giving rise to antibody formation, may actually result in a state in which delayed sensitization cannot be induced; perhaps this is responsible for some cases of so-called "tolerance" or "unresponsiveness." The delayed sensitization process takes place in lymph nodes which drain the site of inoculation and frequently can be prevented by cutting the draining lymphatics or by irradiation or extirpation of these nodes.

From the Department of Bacteriology and Immunology, Harvard Medical School, and Neurology Service, Massachusetts General Hospital.

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*National Neurological Research Foundation Scientist.

2. Circulating antibody appears to bear no relation to the sensitive state. Passive transfer of sensitivity to normal recipients with antiserum is impossible. Delayed hypersensitivity occurs in patients with severe hypogammaglobulinemia, in whom no measurable antibody is synthesized. Special techniques of immunization permit the experimental production of delayed sensitivity in animals in the absence of any detectable formation of humoral antibody.

3. Delayed sensitivity may be largely or completely lost in patients with Boeck's sarcoid, Hodgkin's disease, or certain lymphomas, in whom antibody formation is relatively preserved. This group of diseases may be regarded as producing a specific immunologic defect which is analogous to hypogammaglobulinemia but which affects delayed sensitization rather than antibody formation.

4. The characteristic delayed reaction is made up of perivenous accumulations of lymphocytes and histiocytes, often with some parenchymal destruction in the area infiltrated by these cells. Mild and early lesions do not show polymorphonuclear cell infiltration, hemorrhage, or diffuse necrosis. The evolution of the lesion occurs over several days, rather than in a few minutes or hours.

5. Sensitivity appears to be correlated with the functional activity of circulating, "sensitized" cells, possibly lymphocytes. It is depressed by agents which depress the number of these cells in the circulation, such as x-ray, cortisone, and specific antilymphocyte antisera. It can be passively transferred by transfer of *living* lymphoid cells from sensitized donors to normal recipients, sensitivity being maximal at the moment of transfer and falling off over several days. (When reactions requiring humoral antibody are transferred by transferring cells, sensitivity increases for one or more days as new antibody is synthesized in the recipient.) Essentially identical reactions occur with intravenous or intraperitoneal injection of cells, when antigen is present locally (for example, tuberculin injected intradermally), with local injection of cells and systemic injection of antigen, and finally with local injection of cells mixed with antigen in vitro.

These findings suggest that sensitivity is mediated by circulating, sensitized mononuclear cells, lymphocytes, and/or monocytes, which accumulate where antigen is present, proliferate, and give rise to unknown secondary effects.

Immunologic states which can justifiably be said to possess, in greater or lesser degree, the previously discussed experimental properties include those described in the paragraphs that follow.

1. Tuberculin sensitivity may be properly regarded as the prototype of *infectious allergy*.[1, 2] A number of bacterial infections, such as brucellosis, and many mycotic and viral infections induce delayed sensitivity to certain protein components of the infecting organisms.[3] It is generally recognized that the presence of sensitivity may play an important role in determining the nature of the characteristic lesions of each disease, but this role has never been well evaluated. At one time much emphasis was placed on necrosis as the principal effect, and a very harmful one at that, of the presence of hypersensitivity.[4] Several recent observations lead to a different interpretation. A virus, such as

that which causes measles, is present in lymphoid tissue and respiratory epithelium from the moment of infection. There, as it grows, it produces characteristic changes comparable to the cytopathogenic effects produced in tissue cultures of susceptible cells.[5] The general symptoms and the rash, however, which appear only after the usual immunologic latent period, can be regarded, as von Pirquet insisted half a century ago, as the immunologic response which terminates this infection.[6, 7] It is highly pertinent that, in each of the exanthemas, patients with agammaglobulinemia develop the rash, in the normal manner and usually develop specific immunity to reinfection at the same time.[8] On the other hand, in patients with lymphomas, the measles virus may produce a progressive and ultimately fatal giant-cell pneumonia, while rash and immunity remain conspicuously absent.[9] These findings suggest an important positive role for delayed sensitivity in producing certain types of inflammatory lesion, formerly attributed to the direct action of infection, and also as the basis for immunity to some infectious agents. Nothing definitive can be said about the mechanism of such immunity. As indicated, the delayed reaction consists of a local mobilization of phagocytic cells. These have been shown, in the case of tuberculosis, to possess increased phagocytic power for the antigen-containing bacteria[10] and the ability to inhibit their growth.[11, 12] These properties may cause them to be effective in other infections as well.

2. The *rejection of solid, vascularized homografts* (for example, of skin) has been recognized as immunologic since the pioneer studies of Medawar. Whereas free-floating grafted cells, such as those of an ascites tumor, may be killed by humoral antibody and complement, the evidence is overwhelming that rejection of the usual type of graft is comparable to a delayed reaction.[13] In this instance, a massive infiltration of mononuclear cells occurs in the graft. These cells appear (demonstrated in ordinary histologic sections) to have a direct effect in killing the cells of the graft as well as certain secondary effects via vascular occlusion and ischemia. The finding that donor tissue survives indefinitely (even in a highly immunized recipient), when protected by a Millipore filter with pores too small to permit the access of the recipient's cells,[14, 15] emphasizes the direct role of these cells in the usual graft destruction.

3. In *contact allergy,* allergen applied to the surface of the skin forms antigen by complexing with as yet unidentified constituents of the epidermis.[16] The allergic reaction, then, is a reaction to an artificial component of the surface epidermis. It shows histologically the familiar accumulation of mononuclear cells immediately beneath the epidermis, with invasion and destruction of epidermal cells by them. The mechanism here is indistinguishable from that involved in the destruction of epidermis in a skin homograft. It is important to realize that we are not dealing with a sensitivity of the epidermis itself, since sensitization actually takes place in the draining nodes[17] and since sensitivity can be transferred to normal animals with sensitive lymphoid cells[18] and is controlled by suppression of the circulating lymphocytes.[19] This implies that patch testing with simple chemicals demonstrates a general sensitivity which can, in principle at least, be responsible for producing lesions elsewhere than in the

skin. The sensitivity indicated by positive contact reactions to beryllium salts in patients with various types of beryllium disease,[20] for example, may well lead to the formation of lesions, even in the lung or other viscera where relatively insoluble compounds of beryllium are known to remain for many years. The granulomatous character of visceral beryllium disease depends, perhaps, on the chronicity of the lesion, which is in turn determined by the nature of the allergen. Such disease should be regarded as an *allergic granulomatosis.*

4. Experimental "*auto-allergies*" affecting lens, uvea, myelin of the central and peripheral nervous systems, testis, and thyroid and adrenal glands are readily produced in experimental animals by immunization with homologous, heterologous, or even autologous tissue.[21] There is ample evidence that these entities are the result of delayed sensitization to tissue antigens. The lesions consist of perivenous mononuclear cell infiltrates with destruction of the parenchymal elements containing antigen (for example, myelin); late lesions may show atrophy and scarring. The tissue destruction is formally comparable to that which occurs in graft rejection and in contact allergy. The auto-allergies are, in general, chronic relapsing processes, since the antigens are normal tissue constituents and therefore continually present and since the state of hypersensitivity, which determines the production of lesions, fluctuates with time and may increase following secondary antigenic stimulation. Certain chronic, remittent human diseases which resemble these experimental processes probably have the same mechanism, such as sympathetic ophthalmia and phako-anaphylactic endophthalmitis; with regard to others, such as Hashimoto's disease and multiple sclerosis, the evidence is as yet inconclusive. Another class of human diseases, which includes "postinfectious" encephalomyelitis, neuritis, iridocyclitis, and perhaps mumps orchitis and thyroiditis, while showing an obvious morphologic similarity to the experimental auto-allergies, differs from them in their constant association with viral infections and in their monocyclic course. It is a plausible hypothesis, as yet unsupported by evidence, that these phenomena are in fact allergic reactions of the delayed type to virus. It would be of obvious interest to obtain precise data on the incidence of both categories of human disease in hypogammaglobulinemia and in sarcoid, Hodgkin's disease, and other lymphomas.

It should be stressed here that the "auto-allergy" problem is completely different from the "auto-antibody" problem. All the experimental auto-allergies and their possible human counterparts are accompanied by circulating auto-antibodies against antigens in the diseased tissues. There is no evidence that these antibodies play any role in producing lesions, the crucial test being the failure of passive transfer of disease with serum. Auto-antibodies are present in a variety of chronic destructive tissue diseases, among them such obviously nonimmunologic entities as viral hepatitis, syphilis, and myocardial infarction; on passive transfer, these antibodies do not produce any identifiable lesion. On the other hand, auto-antibodies clearly can produce disease of the various blood elements, there being no evidence for delayed allergy in such processes as acquired hemolytic anemia and idiopathic thrombocytopenic purpura.

5. *Disseminated delayed reactions* to exogenous antigen have been described in guinea pigs[22] and rats[23] injected with mycobacterial adjuvant. The lesions include arthritis, iridocyclitis, subcutaneous nodules, and dermatitis, in which lymphocytic and histiocytic infiltration and granuloma formation, together with tissue destruction, are prominent elements. A variety of evidence has been obtained to show that these lesions are, in fact, delayed allergic responses to antigen(s) of the injected tubercle bacilli, widely disseminated before the appearance of sensitivity. They represent the delayed counterpart of arteritis, glomerulonephritis, and other lesions produced in experimental serum disease, in which humoral antibody is the effective immunologic agency. There is as yet no clear-cut evidence which permits us to identify morphologically similar human entities as having the same immunologic mechanism.

The actual experimental details which justify the description of each of the foregoing entities as being of the delayed allergic type have been omitted in the outlines just given. A single example will serve to illustrate the type of evidence which has been sought in each case. Experimental allergic encephalomyelitis, one of the auto-allergies, is identified as immunologic by (1) the occurrence of a sensitizing event (injection of myelin antigen), (2) a latent period of more than nine days before onset, (3) an anamnestic shortening of the latent period on reimmunization, and (4) the specificity of the response (that is, lesions occur only where myelin antigen is present, largely in the white matter). It is further characterized as delayed by the fact that (1) sensitization can usually be achieved only with the use of adjuvant or of small doses of antigen given intradermally, (2) the histologic picture is one of perivenous mononuclear cell infiltration with varying degrees of parenchymal damage (for example, demyelinization), (3) there is no correlation of disease with circulating antibody and passive transfer of the disease is not possible with antiserum, (4) positive delayed skin reactions to myelin are found and show a clear correlation with the disease process, (5) the disease is suppressed by cortisone or treatment with specific lymphocyte antiserum, and (6) typical encephalomyelitis lesions are produced in normal recipients of living sensitized lymphoid cells. There are a few discrepancies in the observations on certain of the entities described, among them Lawrence's transfer of delayed allergy (in man only) with lysates of blood leukocytes[24] (this is not a true passive transfer, however, since the recipients show a durable, that is, active sensitivity) and the failure of many hypogammaglobulinemic patients to reject skin homografts promptly.[8] The data are otherwise consistent and convincing.

The available experimental facts do not permit a unified interpretation of the mechanism of either sensitization or lesion formation in the group of conditions under discussion. Apparently many or all proteins, including some substances which are very poor inducers of antibody formation, can act as delayed antigens; carbohydrates are not, so far, known to do so. In experiments with purified protein antigens, delayed sensitization is found to appear as early as five days following immunization and may be supplanted after some days or weeks by antibody formation.[25, 26] An anamnestic (booster) injection of antigen

in animals with delayed sensitivity results in disappearance of the sensitivity and the rapid appearance of humoral antibody.[27] It has thus seemed to some workers that a single system of cells in the lymphatic tissue is involved in the two types of response and that delayed sensitization represents an early phase of the cellular events leading to antibody synthesis. However, the failure of carbohydrates to act as delayed antigens (although excellent inducers of antibody formation) and the dissociation of the two immunologic functions in hypogammaglobulinemia and in such diseases as sarcoid seem at variance with this otherwise attractive hypothesis and suggest the possibility that two different families of immunologically competent cells are involved. The effects of immunizing dose and the molecular properties of the antigen which give rise to one type of immunologic response or the other are now under investigation.[28]

With regard to the nature of the delayed reaction itself, in vitro studies have not been helpful. The exposure of sensitized animals' cells, which have been maintained in tissue culture, to antigen may result in either stimulation (peritoneal exudate monocytes)[29] or cell death (fibroblasts, other cell types[?]).[4] To tie together the diverse points presented in this survey, the best suggestion that I can offer is that sensitization involves the production, in lymph nodes draining the inoculation site, of sensitized cells which are released into the circulation. The sensitized cells react to antigen wherever it may be present in the tissues, whether introduced by injection (as in a tuberculin test), artificially formed locally (as by complex formation between a contact allergen and epidermal constituents), or normally present (as in the case of tissue auto-antigens or the genetically determined iso-antigens of a homograft). The reaction consists in part of the local accumulation of these cells after they leave the blood stream through the walls of small veins. The further steps of the process remain to be elucidated. Either the sensitized cells respond to antigen by releasing substances which produce parenchymal damage and/or stimulation of local fixed tissue histiocytes or they are themselves stimulated to develop into histiocytes. Surely there are other possibilities not yet envisioned. The local mass of infiltrating and activated cells and the destruction of parenchymal constituents (epidermis in the case of contact allergy, tissue elements such as myelin or thyroid follicular colloid in the auto-allergies, and all cellular elements in homografts) account for the principal gross and microscopic features of each of these reactions. In any event, we have now reached the point where we can ask the right questions, and we are limited in obtaining full understanding of delayed reactions mainly by the difficulty of cultivating lymphocytes and similar cells in vitro and studying their functional alterations in the presence of antigen.

Let me conclude by calling attention to the importance of recognizing certain categories of disease as immunologic phenomena of the delayed type and others as the results of derangements involving this immunologic function. We must learn to identify and to characterize immunologically (1) human diseases comparable to the experimental auto-allergies, (2) allergic granulomatous diseases due to unmetabolizable chemical substances, and possibly (3) other disseminated lesions due to exogenous (for example, bacterial) antigens. Where

delayed sensitization is lost, as in sarcoid, Hodgkin's disease, and some lymphomas, we must recognize the possibility that familiar infectious agents may produce entirely atypical lesions in which the clinical course is determined by the failure of the usual immune response, and the nature of the lesions is determined exclusively by the cytopathogenic effects of the agents themselves. Finally, where antibody formation is lost, as in agammaglobulinemia, we must anticipate that certain infections or other exogenous agents, which would normally induce antibody production and immunity, may induce delayed sensitivity and the production of disseminated lesions. The high and overlapping individual and familial incidence of lupus erythematosus, rheumatoid arthritis, and agammaglobulinemia might well be investigated from such a point of view.

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IMMUNE MILK IN THE TREATMENT OF HAY FEVER

William B. Sherman, M.D., and Samuel Freedman, M.D., New York, N. Y.

IT IS WELL KNOWN that the human infant receives, through the placenta, antibodies which confer protection against various infectious diseases for which the mother has acquired immunity. The young of a number of other species, including domestic cattle, are born with little or no gamma globulin and without passive immunity. Calves acquire immunity from the mother cow through the colostrum, a special type of milk, containing a high percentage of gamma globulin which is secreted during the first week after delivery.

Extensive studies of bovine colostrum by Peterson and Campbell[1] have shown that it is rich in various antibodies. Their studies indicate that the mammary gland of the cow contains many plasma cells and may act as an organ of antibody formation. Injection of various bacterial antigens, especially species of Salmonella, into the udders during the dry period shortly before the birth of a calf produced agglutinin titers in the colostrum as high as 1:100,000 or 100 times that of the serum of the same cow. When the secretion of colostrum gave way to that of ordinary milk, the titer fell to 1:1,000 but, considering the fact that several quarts were produced daily, the total output of antibodies was tremendous.

In an experiment designed to indicate whether human beings could absorb these antibodies, five adult volunteers whose sera showed no agglutinins for *Salmonella pullorum* drank one quart of milk with an agglutinin titer of 1:1,000 daily for a week. At the end of this period the sera of the volunteers showed titers as high as 1:10.[1]

During 1955 at the University of Minnesota, cows were similarly injected with ragweed pollen and the colostrum was used by Blumenthal[3] to treat patients with hay fever. The results were considered encouraging and, in 1956, the Borden Company injected a series of cows with grass and ragweed pollens and collected colostrum for clinical trials. Some of their material was made available to the allergy clinics of the Roosevelt and Presbyterian Hospitals. Since the material was received at the onset of the ragweed pollen season, the clinical trials were necessarily carried out before any immunologic studies of the colostrum were made. This chance ensured that the therapeutic tests were not influenced by preconceived bias.

From the Allergy Clinics of Roosevelt and Presbyterian Hospitals.
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PREPARATION OF COLOSTRUM

The cows were given 3 weekly injections into each udder during the dry period just before calving. Each injection contained low ragweed pollen, 9.5 mg.; giant ragweed pollen, 9.5 mg.; chenopod, cocklebur, and amaranth pollens, 2 mg. each; and grass pollens, which were not included in our study. The amounts chosen and the use of a mixture of pollen antigens were based on the results obtained by Peterson and Campbell with bacterial antigens. These had shown that the response to one antigen was not affected by others injected simultaneously.

During the first week after calving, colostrum was collected and processed into dried skimmed milk by the usual commercial methods which involve heating to 160°-180° F. (71°-82° C.) for a half hour. The resulting powder was similar in appearance and taste to commercial dried skimmed milk* from untreated cows which was used as a control for the dried colostrum. Both powders were packed in plain envelopes containing the equivalent of a quart of fluid milk and marked only with a lot number, the significance of which was not revealed to the clinic physicians until these tests were completed.

CLINICAL TRIALS

Patients with typical active ragweed hay fever were given one package of the powder a day, equivalent to a quart of milk. All patients included in the series took this amount for at least a week and the majority of them for two or more weeks during the season. Alternate patients were given the immunized and control milks. Each patient was asked to keep a daily written record of the severity of hay fever symptoms. Those who had previously received antihistamine drugs were allowed to use them as necessary but were asked to keep a daily record of the number of pills taken.

The group completing the test included thirty-three patients who had received preseasonal or perennial injections of pollen extract but who were having moderate or severe hay fever at the time they were added to the series, and thirteen patients who had not received previous specific treatment; a total of forty-six patients, twenty-six of whom received the immunized colostrum and twenty who received normal skimmed milk. Many patients were skin tested with ragweed at the start of the milk treatment and again when it was discontinued. Blood for serologic studies was taken from those who had not received pollen injections at the same time.

The results produced by the two batches of material were so similar that, at the end of the test, none of the physicians were able to distinguish which lot was the immunized and which the control milk. On the basis of the records kept, the degree of benefit to each patient was classified on a basis of 0 for no benefit, 1 for slight improvement, and 2 for moderate relief. None reported marked or complete relief. When the scores were totalled, the results

*Starlac, manufactured by the Borden Company, New York, N. Y.

were essentially the same in the groups receiving the immunized milk and the control milk. Actually the average relief for the control group (0.77) was slightly higher than that for the group receiving the treated milk (0.69), indicating that ordinary Starlac was just as effective as the immunized colostrum.

Like many other new medications, this preparation produced a significant number of side effects. Although patients with known allergy or strong aversion to milk were excluded from the series, four patients, or 15 per cent, of the twenty-six taking the immunized colostrum had untoward effects such as diarrhea, abdominal pain, indigestion, and hives. The incidence of side effects in the control group was also 15 per cent or three in twenty patients.

The skin tests with ragweed pollen extract before and after the treatment showed no significant changes.

IMMUNOLOGIC STUDIES

Sera taken before and after at least two weeks of the milk treatment from four patients who had never received pollen injections were tested for blocking antibodies by the passive transfer neutralization method of Loveless.[2] None showed any evidence of blocking activity. Sera from four patients in the control group were similarly tested, also with entirely negative results.

In order to test the colostrum itself for antibodies, the whey fraction was separated from it. This showed no evidence of precipitins for low ragweed pollen extract (80,000 P.N. units per milliliter) in ring tests or the Oudin method.

The whey fraction from the colostrum of the injected cows and that from skimmed milk of normal cows were compared in passive transfer neutralization tests for blocking antibody using the same methods[2] employed for measuring blocking antibody in serum. No blocking antibody was demonstrable in the undiluted whey.

It was thought that the failure to demonstrate antibodies in the colostrum of the treated cows might be due to (1) the use of so many pollens for immunization that the doses of ragweed, the only pollen considered in our study, were inadequate, or (2) that the degree of heat involved in drying the colostrum might have destroyed antibody. During the following spring an attempt was made to avoid these difficulties. Three groups of three cows each were injected during the dry period before calving with low ragweed pollen in amounts comparable to the total of all pollens used in the 1956 series. The udders of three cows were injected with whole ragweed pollen grains, those of three with saline ragweed extract, and those of three others with a glycerinated ragweed extract (52,000 P.N. units per milliliter). The technique and timing of the injections was as described previously. Colostrum from each of these nine cows and from an untreated control cow was collected on the first and fifth days after freshening, quick-frozen in one quart containers, and kept frozen until testing.*

*Courtesy of Protective Foods Corporation, Minneapolis, Minn., and the Borden Company.

Each of these 20 samples of colostrum was tested for blocking antibody by the passive transfer neutralization method previously described. None showed evidence of activity, and no clinical studies were undertaken with these preparations.

DISCUSSION

While both the clinical and laboratory results were clearly negative, and those of the 1956 studies were reported at the meeting of the American Academy of Allergy in February, 1957, publication was delayed at the time in hopes that more effective methods of immunizing the cows might be developed. Since 1957 the authors have had no opportunity for further study of the problem and no immunologic evidence of an improved product has been offered by others. Meanwhile, without the publication of any controlled clinical studies, the use of "protective milk" for the treatment of hay fever has been widely exploited commercially and publicized in popular magazines. Since this promotion has attracted the attention and raised the hopes of many people suffering from allergic diseases, publication of these negative results of controlled studies now seems timely.

SUMMARY

Colostrum collected from cows after injections of ragweed pollen into the udders showed no evidence of precipitating or blocking antibodies for ragweed pollen antigens. A series of twenty-six ragweed hay fever patients treated with the equivalent of a quart of such colostrum daily showed no greater benefits than twenty controls taking an equal amount of commercial skimmed milk.

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AMERICAN ACADEMY OF ALLERGY

HISTORIAN'S REPORT

THE sixteenth annual meeting of the American Academy of Allergy was held at the Hollywood Beach Hotel in Hollywood-by-the-Sea, Florida, Jan. 11, 12, and 13, 1960. A postgraduate course on January 9 and 10 preceded the scientific session. Registration included 284 physician members, 145 physician guests, 118 exhibit personnel, and 184 wives.

The scientific program consisted of 31 original papers and 4 addresses by guest speakers. The appearance of guest speakers for the scientific course and scientific session was made possible in part by a grant from The Merck Sharp & Dohme Postgraduate Program.

The exhibit area had 11 scientific exhibits and 49 commercial exhibits. Presentations were of high caliber and booth personnel were found competent and cooperative.

The Schering Corporation gave an informal cocktail party and reception on Sunday, January 10.

At the annual business meeting on January 12 the following officers were elected:

President: Bram Rose, M.D., Montreal, Quebec, Canada
President-Elect: George I. Blumstein, M.D., Philadelphia, Pa.
Vice-President: Howard Osgood, M.D., Buffalo, New York
Secretary: Joseph W. Noah, M.D., St. Louis, Missouri
Treasurer: James Barnard, M.D., New York, New York
Historian: Sidney Friedlaender, M.D., Detroit, Mich.
Member of Executive Committee: William A. Howard, M.D., Washington, D. C.

The following members were advanced to fellowship: Philip Blank, M.D., Daniel Blitz, M.D., Robert J. Ehrenreich, M.D., Jack E. Goodwin, M.D., Douglas E. Johnstone, M.D., Louis Kreindler, M.D., Gilbert Lanoff, M.D., Wilfred Leith, M.D., Eugene J. Luippold, M.D., Alan L. Michelson, M.D., Charles F. Mohr, M.D., Beverly C. Payne, M.D., Isadore Pitesky, M.D., Ernst O. Schreiber, M.D., Abe Sosman, M.D., Edward L. Strem, M.D., Paul P. Van Arsdel, Jr., M.D., Rudolf E. Wilhelm, M.D., William G. Woodin, M.D.

The following were elected to membership: Gilbert D. Barkin, M.D., Peter P. Barlow, M.D., Jacob Brem, M.D., Dei Jing Chang, M.D., Walter M. Chemris, M.D., Paul Chervinsky, M.D., Hiram D. Cochran, M.D., Evelyn E. Dresner, M.D., Richard C. Field, M.D., Marvin Fuchs, M.D., David M. Glassner, M.D.,

Bernard W. Godwin, Jr., M.D., Carl W. Goebel, M.D., Frederick W. Grover, M.D., Theodore M. Hansen, M.D., Robert M. Heywood, M.D., Rudolf Hoene, M.D., Leon Horowitz, M.D., Robert P. Hudson, M.D., Charles W. Johnson, M.D., Joseph F. Kelley, M.D., Harlin G. Knierim, M.D., Allan Knight, M.D., Thomas A. Lombardo, M.D., E. Donald Lynch, M.D., Stuart A. Mason, M.D., Fred L. Marx, M.D., Albert C. Merkin, M.D., Robert J. Moriarty, M.D., William B. Nevius, M.D., Conrad Nobili, M.D., Ray E. Plymyer, M.D., Robert C. Price, Jr., M.D., Edward Razim, M.D., Emmanuel R. Riff, M.D., Charles Rosen, M.D., Richard S. Shapiro, M.D., Purcell Smith, Jr., M.D., Howard E. Sullivan, Jr., M.D., Thomas M. Thompson, M.D., Stanley M. Vickers, M.D., J. C. Whittier, M.D., Heinz J. Wittig, M.D., Albert Zucker, M.D.

Elected to foreign membership were Oscar P. Guimaraes, M.D., Brazil; James W. Kerr, M.D., Scotland; Franz Kral, M.D., New Zealand; and Audia L. Marino, M.D., St. Louis, Missouri.

The following were elected to affiliate fellowship: Douglas O. Cross, M.D., Edward L. Foubert, Jr., Ph.D., Julio A. Moretti, M.D., Edward G. Sayers, M.D., and Pavao Stern, M.D.

The consensus of persons attending the annual meeting was that it presented an excellent scientific program with a fine climatologic influence resulting in one of our best annual meetings.

The seventeenth annual meeting of the Academy will be held Feb. 6, 7, and 8, 1961, at the Statler-Hilton Hotel in Washington, D. C.

Respectfully submitted,
Sidney Friedlaender, M.D., *Historian*

OBITUARIES

ROBERT ANDERSON COOKE

Robert Anderson Cooke, Honorary Fellow of The American Academy of Allergy and a pioneer in the field of allergy, died in the Roosevelt Hospital, New York City, on May 7, 1960.

Dr. Cooke was born on Aug. 17, 1880, in Holmdel, N. J. He received the A.B. degree in 1900 and the M.A. degree in 1904 at Rutgers College, and the M.D. degree from Columbia University in 1904. He was the fourth generation physician in his family. The honorary degree of Doctor of Science was conferred on him by Rutgers College in 1925, where he served for years as trustee.

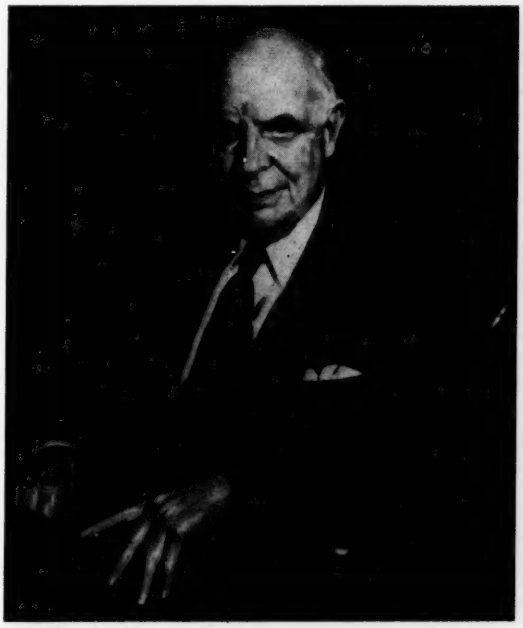
After internship at the Presbyterian Hospital, New York City, he served for years as attending physician at the City, Bellevue, Presbyterian, and Post-Graduate Hospitals. He taught applied immunology and clinical medicine and allergy at the Cornell University Medical School for twenty years, until 1940. He was Consulting Physician to numerous hospitals in lower New York State. He served on the Editorial Board of the Journal of Allergy and of the Annals of Internal Medicine for many years. During World War II, he was a civilian consultant to the Secretary of War under the Surgeon General of the Army. He was long an active member of the Advisory Board for Medical Specialties; he was Chairman of the Science and Education Council of The Allergy Foundation of America.

Dr. Cooke was a founder of The American Society for the Study of Asthma and Allied Conditions, one of the two parent societies of The American Academy of Allergy. The germination of the idea of forming this Society occurred in March, 1923, when Dr. Cooke, Dr. F. M. Rackemann, Dr. George M. MacKenzie, and Dr. Ross Golden each read a paper on asthma at a meeting of the Medical Society of the County of New York, with discussion by Dr. James Alexander Miller and Dr. Harry L. Alexander. Subsequent correspondence led to a meeting, on March 7, 1924, in Dr. Cooke's office in New York at which the Society was formed, with Dr. Cooke as its first President, Dr. Rackemann as Secretary, and Doctors Warfield T. Longcope, Rackemann, and MacKenzie as its Executive Committee. The First Annual Meeting of the Society was held in Atlantic City on May 4, 1924. Dr. Cooke also founded The Association of Allergy Clinics of Greater New York.

Dr. Cooke was a Fellow of The American College of Physicians (successively Governor, Regent, First Vice-President, and Master). He was a member of The American Association of Immunologists, the Society for Clinical Investigation, the Clinical and Climatological Association, the Association of American

Physicians, the American Association for the Advancement of Science, the American Academy of Science, the New York Academy of Medicine, the Harvey Society, Delta Kappa Epsilon, the American Medical Association, and of his State and County Medical Societies. He was an Honorary Member of the International Association of Allergology and of the national allergy societies of Argentina, Brazil, Cuba, France, and Spain.

Dr. Cooke was himself an allergic individual, having been subject to asthma in childhood on exposure to animals on his father's farm. As an intern he nearly died of an anaphylactic shock induced by an injection of diphtheria antitoxin. In 1910, after having been in practice a few years, he began to devote himself to the study and practice of the infant field of allergy, and published his first paper *The Treatment of Hay Fever by Active Immunization* in 1915.



ROBERT ANDERSON COOKE

After some earlier work in allergy at the Post-Graduate Hospital, he established, in February, 1919, his world-renowned clinic and laboratory devoted to allergy at the New York Hospital; Dr. Albert Vander Veer and Dr. Arthur F. Coca were associated with him in this beginning. Dr. Aaron Brown, Dr. Will C. Spain, Dr. Matthew Walzer, Dr. Sanford B. Hooker, Dr. Richard A. Kern, and Dr. Leslie N. Gay were connected with this clinic in its early stages. Later, most of these men established allergy clinics elsewhere. During the succeeding forty years, very many men, now leaders in the field of allergy, secured their training under Dr. Cooke, going thence to carry on clinics and to teach and practice allergy in the United States and abroad. Dr. Cooke's clinic and laboratory were removed in 1932 to the Roosevelt Hospital, New York City, where in 1950 it became the Institute of Allergy, with Dr. Cooke as Director.

Dr. Cooke was a dedicated and inspired teacher. In this new and growing field of medicine, in which empiricism and pure clinical experience were beginning to play so large a role, Dr. Cooke held steadfastly to the scientific and experimental approach. His attitude did as much as that of any other early worker in the field to build up a solid basis of experimental facts underlying our present knowledge of clinical allergy. He and his co-workers published many articles on research. Among the more important areas of discovery, were the demonstration of the "blocking antibody" in allergic patients who had undergone injections with their specific allergens, and studies on the chemical standardization of extracts of allergens, which culminated in the widely used protein nitrogen basis (so-called "Cooke Unit") for standardizing many extracts. In 1947, Dr. Cooke published the book *Allergy in Theory and Practice* which is a standard textbook.

In the American Academy of Allergy, Dr. Cooke had served on its Advisory Committee and as Chairman of its Committee on Education. He was a regular attendant at its Annual Meetings and appeared innumerable times on the scientific program. He was elected to Honorary Fellowship in 1953 in recognition of his many contributions to the knowledge of allergy and of his years of service to the Academy. At the 16th Annual Meeting of the Academy in Florida on Jan. 11, 1960, Dr. Cooke was scheduled to present the First William H. Schultz (Schultz-Dale technique) Lecture, but unfortunately, illness prevented him from being present. His subject was "Research in the Field of Allergy" and this lecture appeared in the May-June, 1960, issue of the JOURNAL OF ALLERGY.

Dr. Cooke held worldwide recognition as an eminent pioneer and continuing leader in the field of allergy. During his lifetime he exercised a very great influence over the development of the study and practice of allergy both here and abroad. In his going, the world has lost an indefatigable worker and an honest and inspiring preceptor. The Academy has lost one of its most honored and devoted members.

The Academy extends its most sincere sympathy to his son, Robert A. Cooke, Jr., to his three grandchildren, to his brother Edward, and to his two sisters, the Misses Sarah and Maria Cooke.

Howard Osgood

KURT MANSBACHER

KURT MANSBACHER died in St. Louis, Missouri, on Dec. 20, 1958.

Dr. Mansbacher was born in Dortmund, Germany, on Jan. 16, 1895. He attended the University of Heidelberg and the University of Berlin, graduating from the latter in 1916, and then received his M.D. degree from the University of Munich in 1919. His internship was served at Dortmund City Hospital, 1919-1921, followed by residency training in the City Hospitals in Berlin in 1921 and 1922. He was licensed to practice in the State of Missouri in 1934 and took up his residence in St. Louis in the same year. In 1940, Dr.

Mansbacher became Instructor in Clinical Medicine at the Washington University School of Medicine. He was associated with the staffs of the Jewish, Barnes Memorial, and City Hospitals in St. Louis. He became a member of the American Academy of Allergy in 1956 and was also a member of the American Medical Association and of the St. Louis Medical Society.

He is survived by his wife, Ilse Mansbacher, and by a brother, Ernest.

Joseph W. Noah

JONATHAN ZONIS

JONATHAN ZONIS, allergist of Boston, Mass., died on July 18, 1959, of an acute coronary occlusion.

Dr. Zonis attended Harvard College, and received his M.D. degree cum laude from Tufts Medical School in 1924 after which he interned at Buffalo City Hospital, Buffalo, New York. He then entered general practice in Boston, and was also a teacher of bio-chemistry at Tufts Medical School. He later became assistant chief-of-staff at the Jewish Memorial Hospital. He was also a member of Norfolk District Medical Society, Massachusetts Medical Society, and the American Medical Association.

Seventeen years ago, he entered the field of allergy by serving a preceptorship in allergy with Dr. Milton Cohen at the Asthma and Hay-fever Clinic, Cleveland, Ohio. He also studied at the Division of Allergy, Columbia University, New York Post-Graduate Medical School. He became a member of the American Academy of Allergy, International Correspondence Society of Allergy, and a fellow in the American College of Allergy. His office for the practice of allergy was on Commonwealth Avenue, Boston, Mass. For thirty years he was on the staff at Beth Israel Hospital, Boston, Mass., and served in the Allergy Clinic there.

Although a specialist in allergy for many years, he never lost the sympathetic interest and the informal, friendly approach of the family practitioner. The devotion and love that his patients held for him were in direct proportion to the service and personal interest he devoted to them.

Jonathan Zonis was a modest and unassuming person. He was marked with kindness, gentleness, humility, and a sense of humanity that endeared him to all. He never sought fame or glory, and his bigness was his integrity and the manner in which he lived his life.

Dr. Zonis was also a member of the Massachusetts Physicians Art Society and frequently exhibited his paintings at medical society meetings.

He is survived by his beloved wife, Frances, a son, Dr. Melvin Zonis, Pediatrician and Allergist of Boston, Mass., and a daughter, Mrs. Jerome Listernick.

His passing represents a considerable loss to his family, friends, and patients.

F. Z.

ANNOUNCEMENTS

Buffalo Allergy Society

The Buffalo Allergy Society announces that its new officers for 1960-61 are:

President, Thelma Brock, M.D., 964 Delaware Avenue, Buffalo 9, N. Y.

Secretary, Leonard Wolin, M.D., 1201 Colvin Avenue, Buffalo 23, N. Y.

Treasurer, George B. Rosenfeld, M.D., 442 Beach Road, Cheektowaga 25, N. Y.

Michigan Allergy Society

The Michigan Allergy Society announces the election of the following officers for the year 1960-61:

President, Robert G. Lovell, M.D., Ann Arbor.

Vice-President, Alex S. Friedlander, M.D., Detroit.

Secretary, Hilda M. Hensel, M.D., Monroe.

Treasurer, Israel Wiener, M.D., Detroit.

Midwest Allergy Forum

The Annual Meeting of the Midwest Allergy Forum will be held at the Penn Sheraton Hotel, Pittsburgh, Pennsylvania, Oct. 22 and 23, 1960.

The program will include a panel discussion on Chronic Urticaria, Drug Allergy, and Repository Therapy, in addition to presentations on bronchial asthma.

University of Tennessee

A postgraduate course in allergy will be given by the University of Tennessee, Memphis, Tennessee, November 3 and 4, 1960.

For further details write to the Post-Graduate Department, University of Tennessee, 62 S. Dunlap Street, Memphis 3, Tenn.